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THE ROLE OF SCL IN HAEMATOPOIESIS AND LEUKAEMOGENESIS

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A thesis submitted for the degree of Doctor of Philosophy of the Open University

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"Omnis cellula a cellula" (every cell comes from a cell)

Rudolf Virchow

"La vraie générosité envers l'avenir consiste à tout donner au présent"

Albert Camus

"They are shallow animals, having always employed their minds about Body and Gut, they imagine that in the whole system of things there is nothing but Gut and Body" (on doctors)

Samuel Taylor Coleridge (1796)

ABSTRACT

The fundamental question of how restriction of differentiation potential and activation of lineage-specific gene expression in progenitor cells are regulated is at the heart of research into the molecular mechanisms of biology. The basic helix-loop-helix (bHLH) transcription factor SCL/TAL-1 serves as a paradigm for a transcription factor with pivotal roles in haematopoietic stem cell (HSC) specification, maturation of haematopoietic lineages as well as T-cell leukaemogenesis. Interestingly, it has been described as both a transcriptional activator and a repressor. However, the molecular mechanisms conferring these opposing functions are currently unknown.

As a first step towards characterising the role of SCL in haematopoiesis and leukaemogenesis, we performed a gain-of-function study using a transgenic mouse model over-expressing SCL. Although no leukaemia was observed, constitutive expression of SCL or a mutant lacking the DNA binding domain in HSCs and early progenitors caused a severe defect in T- and B-lymphopoiesis. This was likely due to an interaction with the important lymphoid regulator and SCL heterodimerisation partner E2A.

Then, we set up a proteomics strategy to identify protein partners of SCL in haematopoiesis. We isolated SCL-containing protein complexes in erythroid cells and megakaryocytes using a novel purification strategy based on *in vivo* biotinylation of SCL. Mass spectrometry analysis led to the identification of several previously unknown candidate partners including ETO-2, a member of

the ETO family of co-repressor proteins and SSDP2, a partner of LDB-1. Using several different approaches, we validated the SCL/ETO-2 interaction in cell lines as well as primary erythrocytes and megakaryocytes. We then identified Gfi-1b, a critical regulator of erythropoiesis with repressive function and the co-repressor mSin3A as additional partners of SCL and ETO-2 in erythroid cells, but not megakaryocytes. Further characterisation in transactivation experiments and during *in vitro* differentiation of primary fetal liver cells led us to hypothesise that, in early erythropoiesis, ETO-2 might confer a repressive function to SCL.

In conclusion, this study gives a first insight into how the composition of the multimeric protein complexes including SCL might modulate haematopoietic differentiation and cell fate decision.

ABBREVIATIONS

| | |
|--------|--|
| AA | aminoacid |
| Ab | antibody |
| AGM | aorto-gonad-mesonephros |
| BFU-E | burst forming unit-erythroid |
| BFU-MK | burst forming unit-megakaryocyte |
| BHLH | basic helix-loop-helix |
| cDNA | complementary DNA |
| CFU-E | colony forming unit-erythroid |
| CFU-E | colony forming unit-erythroid |
| CFU-MK | colony forming unit-megakaryocyte |
| CLP | common lymphoid progenitor |
| CMP | common myeloid progenitor |
| Co-IP | co-immunoprecipitation |
| cpm | counts per minute |
| dCTP | 2'-deoxycytosine 5'-triphosphate |
| DLP | dorsal lateral plate |
| DMSO | dimethylsulphoxide |
| DNA | deoxyribonucleic acid |
| DNase | deoxyribonuclease |
| dNTP | 2'-deoxynucleotides 5'-triphosphate |
| DTT | dithiothreitol |
| EDTA | ethylenediaminetetraacetic acid |
| EPO | erythropoietin |
| ES | embryonic stem cell |
| FCS | Foetal calf serum |
| GMP | common granulocyte-macrophage progenitor |
| HAT | histone acetyl transferase |
| HSC | haematopoietic stem cell |
| HDAC | histone deacetylase |
| ICM | intermediate cell mass |

| | |
|--------------------|--|
| IN | input |
| IP | immunoprecipitation |
| IPTG | isopropyl β -D-thiogalactopyranoside |
| kb | kilo-base |
| kDa | kilo-Daltons |
| MEL | mouse erythroleukaemia |
| MEP | common erythro-megakaryocyte progenitor |
| mg | milligram |
| min | minute |
| ml | millilitre |
| mM | millimolar |
| MOPS | 3-[N-morpholino]propanesulphonic acid |
| mRNA | messenger RNA |
| nmol | nanomole |
| ON | overnight |
| $^{\circ}\text{C}$ | degrees centigrade |
| PAS | paraaortic splanchnopleura |
| PBS | phosphate-buffered saline |
| PCR | polymerase chain reaction |
| PD | pull-down |
| pmol | picomole |
| RNA | ribonucleic acid |
| rpm | revolutions per minute |
| RPMI | Rosswell Park Memorial Institute |
| RT | room temperature |
| RT-PCR | reverse transcription-PCR |
| SCL | stem cell leukaemia gene |
| SDS | sodium dodecyl sulphate |
| sec | second |
| TBE | Tris-Borate-EDTA |
| TCR | T cell receptor |

| | |
|---------------|---|
| TE | Tris-EDTA |
| TPO | thrombopoietin |
| Tris | 2-amino-2-(hydroxymethyl)-1,3-propanediol |
| Tween | polyoxyethylene sorbitan monolaurate |
| U | unit |
| UN | unbound |
| UV | ultraviolet |
| V | volt |
| VBI | ventral blood island |
| μg | microgram |
| μl | microlitre |
| μM | micromolar |
| YS | yolk sac |

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CHAPTER I : GENERAL INTRODUCTION

1 Haematopoiesis as a model for cell commitment and differentiation

During development and adult life, commitment and differentiation from a pluripotent progenitor cell to a lineage-restricted mature cell are modulated by the microenvironment, cellular interactions and the combined action of numerous signaling molecules and transcription factors. These processes involve progressive restriction of the differentiation potential of the progenitor cells and acquisition of lineage-specific patterns of gene expression by their progeny.

The haematopoietic system serves as an ideal model for studying the molecular and genetic basis of such a differentiation process. Blood lineages, which all derive from a pluripotent progenitor called the haematopoietic stem cell (HSC), are divided into two main groups : the myeloid and the lymphoid compartments (figure 1.1, differentiation). The former group consists of the red cells, megakaryocytes/platelets, granulocytes, basophils, eosinophils and monocytes/macrophages. The latter group comprises the T, B and NK (natural killer) cells. The haematopoietic system has to sustain high productivity and high diversity and needs to adapt to physiopathological conditions in order to keep the number of blood cells constant. The analysis of this tightly regulated process called haematopoiesis has been instrumental in elucidating the mechanisms underlying cell fate decision and cellular maturation.

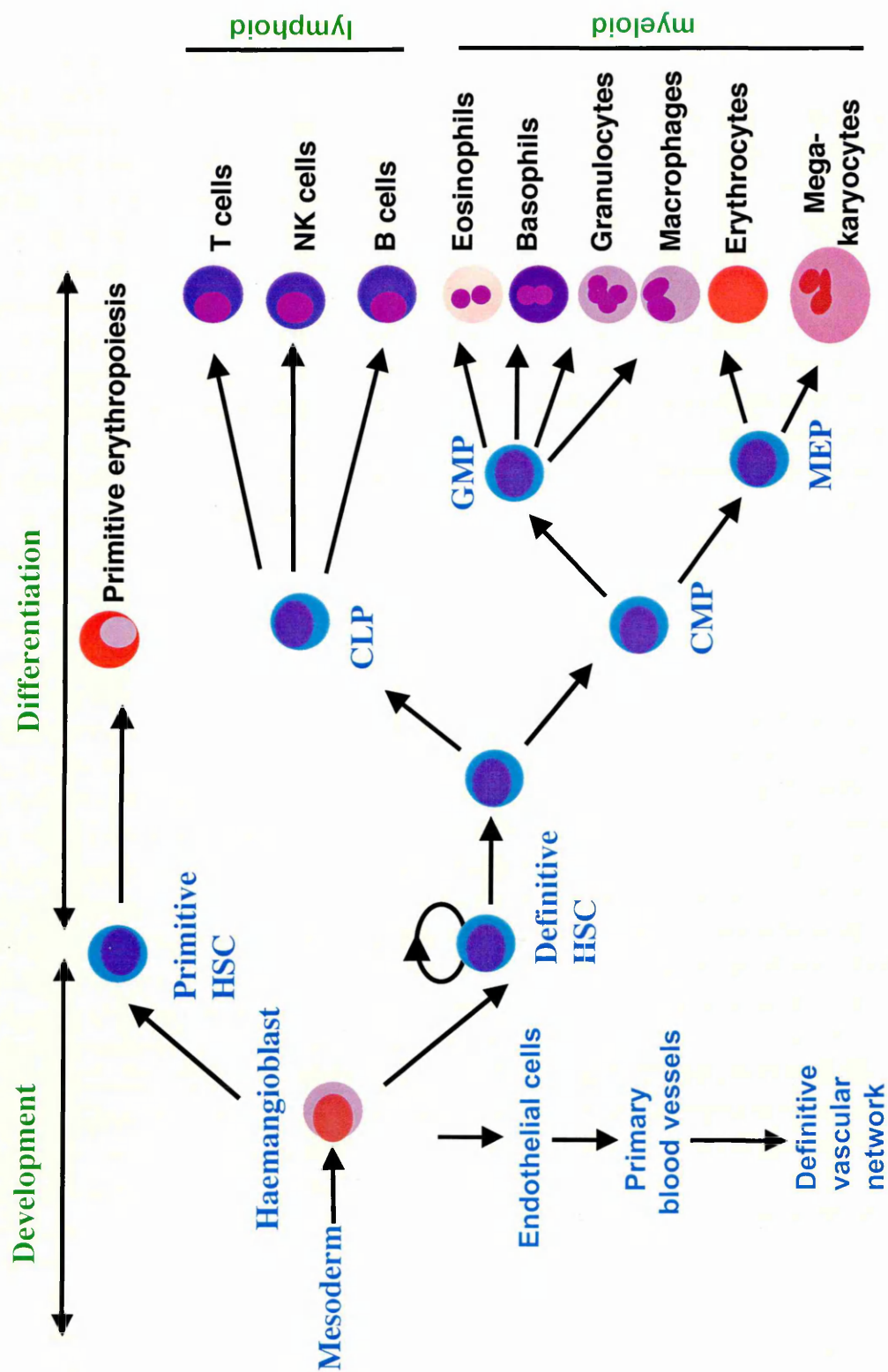


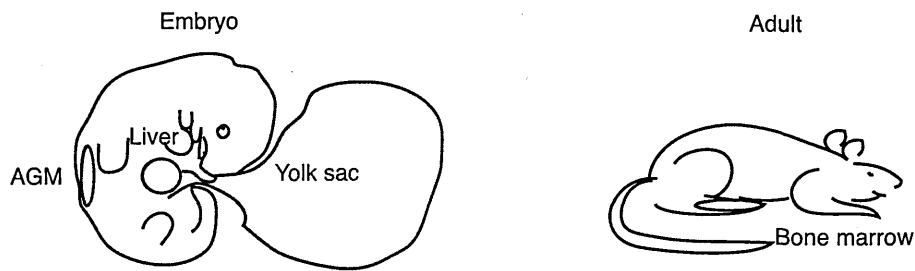
Figure 1.1: Cascade of events leading to production of primitive and definitive haematopoietic and endothelial lineages from a common mesodermal precursor, the haemangioblast

1.1 Haematopoiesis and ontogeny

Haematopoiesis is a highly conserved process in vertebrates¹ and invertebrates and occurs at distinct anatomical sites during development. Studies of haematopoiesis in mammals (mouse), birds (chicken, quail) as well as lower vertebrates (zebrafish and xenopus) and insects (drosophila) have greatly contributed to our understanding of haematopoietic development and it has become apparent that there are two phases of haematopoiesis during ontogeny in these organisms (figure 1.2). Below, I have chosen a few examples that illustrate the high degree of conservation of haematopoietic development during evolution.

Blood cells are specified from mesodermal precursors and are produced through a first wave of primitive (embryonic) haematopoiesis, which begins in the extra-embryonic yolk sac (YS) blood islands in higher vertebrates and consists mainly of nucleated red cells² (figure 1.1 and 1.2). The equivalent region in zebrafish (reviewed in³) is located intraembryonically and is known as the intermediate cell mass (ICM)³. In drosophila (reviewed in⁴), this embryonic phase of haemopoiesis is initiated by a population of hemocytes originating from the head mesoderm and by crystal cells near the anterior region of the gut⁵.

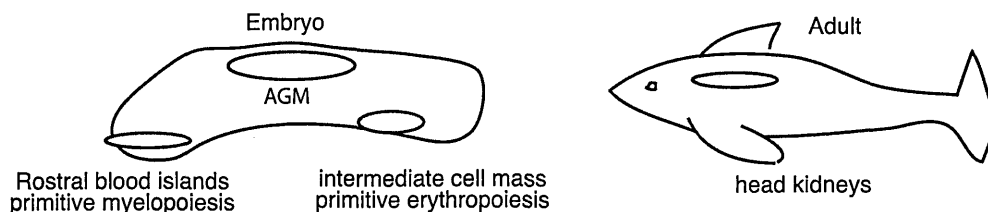
The first evidence suggesting that there was an independent intra-embryonic site for generation of definitive (adult) haematopoietic cells came from experiments performed by F. Dieterlen-Lievre and colleagues on chicken-quail chimeric embryos⁶. They showed that YS progenitors failed to produce definitive haematopoiesis but that an intra-embryonic region contained haematopoietic precursors able to generate blood cells found throughout life. These observations were then confirmed in other species. In mice, for example, before circulation is



A Schematic presentation of murine blood cell ontogeny

Primitive haematopoiesis is initiated in the yolk sac at 7.5 dpc and progressively replaced by by 9.5 to 11.5 dpc by definitive haematopoiesis arising from the AGM and by 12.5 dpc the fetal liver. In the adult animal, the main site of haematopoiesis is the bone marrow. AGM= aorta-gonad-mesonephros region

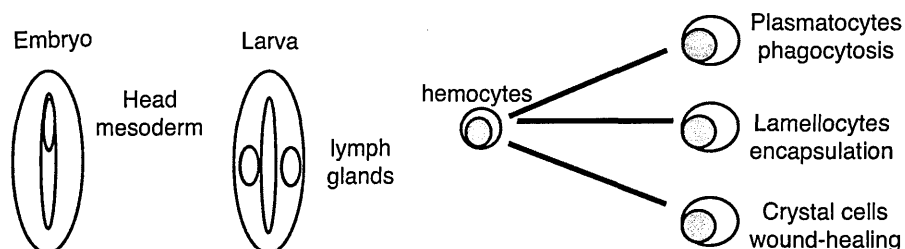
Zebrafish



B Schematic presentation of Zebrafish blood cell ontogeny

In Zebrafish, the primitive sites of haematopoiesis starting from 12 hpf are the intermediate cell mass (ICM) and the rostral blood islands. Around 24 hpf this site is replaced by the AGM equivalent in the dorsal aorta. Adult haematopoiesis is initiated 4 dpf in the head kidneys. h/dpf= hours/days post fertilisation

Drosophila



C Schematic presentation of Drosophila blood cell ontogeny and haematopoiesis

In Drosophila, the first site of embryonic haematopoiesis is the head mesoderm. In early larval development haematopoiesis shifts to the lymph glands (left panels). Similar to the hierarchy of blood cell development in higher organisms, Drosophila blood cells arise from early precursors called hemocytes that will give rise to various haematopoietic lineages.

Figure 1.2 Overview of the ontogeny of haematopoietic cells

established, YS progenitors do not generate multipotent haematopoietic cells in *in vitro* assays whereas cells produced in the intra-embryonic aorta-gonadomesonephros region (AGM, also called paraaortic splanchnopleura (PAS) at earlier developmental stages) do so^{7,8}. It is now generally accepted that the transient wave of primitive erythropoiesis occurring at around 7.5 dpc (days post-coitum) is progressively replaced, by 9.5 to 11.5 dpc, by the second wave of definitive haematopoiesis first developing within the PAS/AGM and ensuring the generation of adult myeloid and lymphoid cells. As primitive haematopoiesis declines and circulation is established, cells produced in the the AGM are thought colonize the fetal liver (and to a much lesser extent the yolk sac) at around 11 dpc, which becomes the main site of blood production in mid- to late-gestation embryos⁹. Towards the end of gestation, another shift occurs and the bone marrow becomes the predominant site of definitive adult haematopoiesis (figure 1.2, top). Studies of haematopoietic development in *Xenopus* have confirmed the dual origin of embryonic and adult blood. In *Xenopus* embryos, the ventral blood island region (VBI, the YS equivalent) and the dorsal lateral plate (DLP) (aorta-gonadomesonephros region (AGM) equivalent) derive from different blastomeres. In an elegant study involving lineage tracing, Ciau-Uitz et al. have shown that aortic clusters (or adult HSCs) derive from the blastomere that gives rise to the DLP¹⁰. In zebrafish, haemopoiesis moves from the ICM to the ventral wall of the dorsal aorta (AGM equivalent) before establishing itself in the adult kidney (figure 1.2, middle panel). In *drosophila*, the lymph glands become the main site of haemopoiesis at the late larval stages (figure 1.2, bottom panel).

1.2 Origin of the haematopoietic stem cell: search for the haemangioblast

In recent years, considerable effort has been put into trying to define the origin of the haematopoietic stem cell (HSC). The close spatial relationship between haematopoietic cells and angioblasts in both the extra-embryonic and embryonic mesoderm and the fact that endothelial and haematopoietic precursors share a number of transcription factors and surface markers suggest the existence of a common progenitor, the putative haemangioblast^{11,12}(Figure 1.1, development). Indeed, targeted disruption of an endothelial marker in the mouse (vascular endothelial growth factor (VEGF) receptor-2, *flk1*¹³) and mutation of the zebrafish *cloche* locus affect development of both blood and endothelial lineages¹⁴. Besides, several groups¹⁵⁻¹⁷ were able to show in *in vitro* differentiation assays that an embryonic stem (ES) cell-derived progenitor (called blast-colony forming cell) can produce adherent endothelial cells, as well as primitive and definitive erythroid cells, macrophages and neutrophils, thus confirming *in vitro* the existence of a common endothelial-haematopoietic precursor. However, until recently, the haemangioblast had not been isolated in vertebrate embryos, although several studies suggested that endothelial cells isolated from the lining of dorsal aorta and the AGM region possessed haematopoietic stem cell activity¹⁸⁻²¹. Since then, Huber et al have demonstrated the existence of cells displaying both vascular and haematopoietic activity in the embryo proper²². It is now generally accepted that clusters of HSCs appear on the floor of the dorsal aorta by budding from a hemogenic endothelium. In zebrafish, overlapping expression patterns of blood and endothelial genes in the posterior lateral mesoderm (PLM) also suggest the existence of haemangioblast-like cells²³⁻²⁵.

1.3 Functional and phenotypic analysis of HSCs

The earliest cell to be defined in the highly hierarchised haematopoietic system is the pluripotent HSC (Figure 1.1). This cell has been characterised both functionally in transplantation experiments and immunophenotypically using cell surface markers and dyes. Thus, it was defined by its long-term reconstitution capacity of all haematopoietic lineages in serial and competitive bone marrow transplantation experiments using tertiary recipients^{26,27}. Only definitive HSCs originating from the PAS/AGM, but not primitive HSCs produced in the YS, can reconstitute bone marrow haematopoiesis in adult recipients²⁸.

In addition, the phenotypic specification of this earliest adult haematopoietic stem cell has become more and more sophisticated. The murine $c\text{-kit}^+ \text{Lin}^- \text{Sca}^+$ (KLS) population clearly contains most of the cells with HSC potential. This KLS population can be further divided into $\text{CD38}^+ \text{CD34}^+$ and $\text{CD38}^+ \text{CD34}^{\text{low}}$ cells. $\text{CD38}^+ \text{CD34}^+$ are responsible for the immediate radioprotection of lethally irradiated mice, whereas $\text{CD38}^+ \text{CD34}^{\text{low}}$ cells have long-term reconstitution capacity for both myeloid and lymphoid cells²⁹ and constitute the bone marrow stem cell pool. Besides, this HSC population effectively exports the vital dye Hoechst and represents the side population in the flowcytometric forward-sideward scatter³⁰.

1.4 The precursor compartment and mature blood cells

The HSC gives rise to partially committed multipotent precursors, also called common lymphoid progenitor (CLP) and common myeloid progenitor (CMP). The CLP generates B, T and NK cells whereas the CMP give rises to the megakaryocyte-erythrocyte progenitor (MEP) and the granulocyte-monocyte

(GMP) progenitor at the origin of the macrophage/monocyte, granulocyte/neutrophil, basophilic and eosinophilic lineages (see Figure 1.1).

In the following paragraph, I wish to briefly outline the steps involved in the differentiation of the CLP and the MEP into their respective lineages.

Lymphopoiesis

Lymphocyte development occurs through a common lymphoid progenitor (CLP), which has restricted lineage potential. Thus, isolation of CD10+/CD19- lymphoid progenitors from bone marrow revealed that these cells were capable of developing into T, B, and NK, or lymphoid dendritic cells, but not into myeloid or erythroid cells³¹. Moreover, Kondo et al showed convincingly that a single cell of a rare population of interleukin 7 receptor expressing bone marrow cells can give rise to T and B, but not myeloid cells³². CLPs develop into three distinct cell types : B and T lymphocytes and NK cells. Beginning with lineage commitment and continuing throughout differentiation, both B and T lymphocytes develop through stages that can be defined by the rearrangement of antigen receptor genes, the acquisition or loss of cell surface and intracellular proteins, and responses to growth and survival factors (see chapter III and figure 3.1).

A fundamental difference between mammalian B and T cell development lies in the anatomical site in which it occurs. B cell development can be divided into two stages : an antigen-independent stage that occurs primarily in fetal liver and bone marrow, and an antigen-dependent stage that occurs in secondary lymphoid organs. T cell development on the other hand consists of an essential progenitor migration step from fetal liver or bone marrow to the thymus. The precise phenotype of this migratory progenitor is unknown and relatively little is known regarding the mechanisms that attract marrow lymphoid progenitors to the thymus,

but chemotactic factors produced by the thymic microenvironment probably play a role³³. Although historical views have assumed that the thymus only functions in young humans and mice because of its well-known involution, this viewpoint has been seriously challenged and it now appears that the T cell pool is replenished via a functional thymus throughout life³⁴.

Erythropoiesis

The existence of a common megakaryocyte/erythroid precursor was first suggested upon study of erythroleukaemic and megakaryoblastic cell lines showing co-expression of erythroid and megakaryocytic markers³⁵⁻³⁷. Subsequently, bipotent progenitors were characterised in culture assays³⁸ and isolated by virtue of their cell surface phenotype³⁹⁻⁴¹.

Erythropoiesis is a complex multistep process encompassing the differentiation of the MEP to mature erythrocytes⁴² (figure 1.5 A, B). The steps involved in this process include the differentiation to early erythroid progenitors (burst-forming units-erythroid, BFU-E), then to late erythroid progenitors (colony-forming units-erythroid, CFU-E) and finally to the morphologically recognisable erythroid precursors. A key event of the late stages of erythroid differentiation is nuclear condensation, followed by extrusion of the nucleus to produce enucleated reticulocytes and finally mature erythrocytes. As differentiation proceeds, haemoglobinisation of the cells increases, cells express Ter119 and downregulate the surface marker molecule CD 71.

A model to study erythroid differentiation

The Murine Erythroid Leukaemia (MEL) cell line serves as a practical model for erythroid differentiation. It was first derived from murine proerythroblasts transformed with the Friend leukaemia virus⁴³. Morphologically, these cells have

some of the characteristics of immature erythroblasts, but they can be induced to initiate erythroid differentiation by the addition of dimethylsulfoxide (DMSO) to the culture medium⁴⁴. Globin mRNA can be detected after 2 days of induction with a maximum peak after 4 days. Around this time, cells that stain positive for haem appear⁴⁵.

Megakaryocytopoiesis

The study of megakaryocytopoiesis has been particularly difficult because of the relative infrequency of megakaryocytes in the bone marrow and their fragility *in vitro*. However, it is now generally accepted that megakaryocytes derive from the MEP (see above). This is also supported by the close relationship between early erythroid and megakaryocyte differentiation at the molecular level⁴⁶. The transition between multipotent precursor and megakaryocyte-restricted progenitors involves expression of the *c-mpl* oncogene or thrombopoietin receptor^{47,48}. The earliest definable committed progenitor, the megakaryocytic burst forming cell (BFU-MK) gives rise to the GPIIbGPIIIa positive megakaryocytic colony forming cell (CFU-MK), which eventually stops mitosis and enters endomitosis leading to production of polyploid precursors with scant cytoplasm (figure 1.6 A, B). These cells produce Platelet factor 4 (PF4) and von Willebrandt Factor (vWF). Upon completion of endomitosis, the cells have acquired a DNA content of up to 128N (where 2N is the DNA content of a somatic cell), develop a mature cytoplasm and eventually start to release platelets. An individual mature megakaryocyte produces in the order of 2000 to 3000 platelets.

A model to study megakaryocyte differentiation

L8057 is a murine megakaryoblastic cell line derived from irradiated mice. This cell line can be induced to differentiate along the megakaryocyte/platelet

lineage expressing increased levels of the megakaryocyte specific enzyme acetylcholinesterase, megakaryocytic/platelet-specific surface antigens such as GPIIb, and showing increased ploidy following incubation with phorbol diester⁴⁹. Thus, L8057 cells provide a cellular model for studying the characteristics of megakaryocytic differentiation.

2 MOLECULAR EVENTS UNDERLYING THE PROGRESSIVE DIFFERENTIATION PROGRAM OF HAEMATOPOIETIC CELLS

Haematopoietic cells face at least four choices during their differentiation process (Figure 1.3). They can self-renew or proliferate, differentiate to more committed precursors, undergo apoptosis or enter quiescence. Clearly, the regulators and molecular events that can influence these decisions are likely to play critical roles during normal haematopoietic differentiation.

2.1 Differentiation of the haematopoietic stem cell: instructive or stochastic model ?

It is now generally accepted that the proliferative and self-renewal capacity of the adult HSC declines with differentiation. At the same time, lineage choice becomes more and more restricted as highly specialised transcriptional programs are switched on. Thus, the modern concept of « stemness » implies that, in contrast to terminally differentiated cells, the haematopoietic stem cell is still receptive to multiple fate decisions. Although much progress has been made, the molecular mechanisms underlying the progressive commitment and differentiation of haematopoietic cells remain to be fully elucidated. Two contrasting lines of thought dominate the current view of what directs cell fate decisions: the

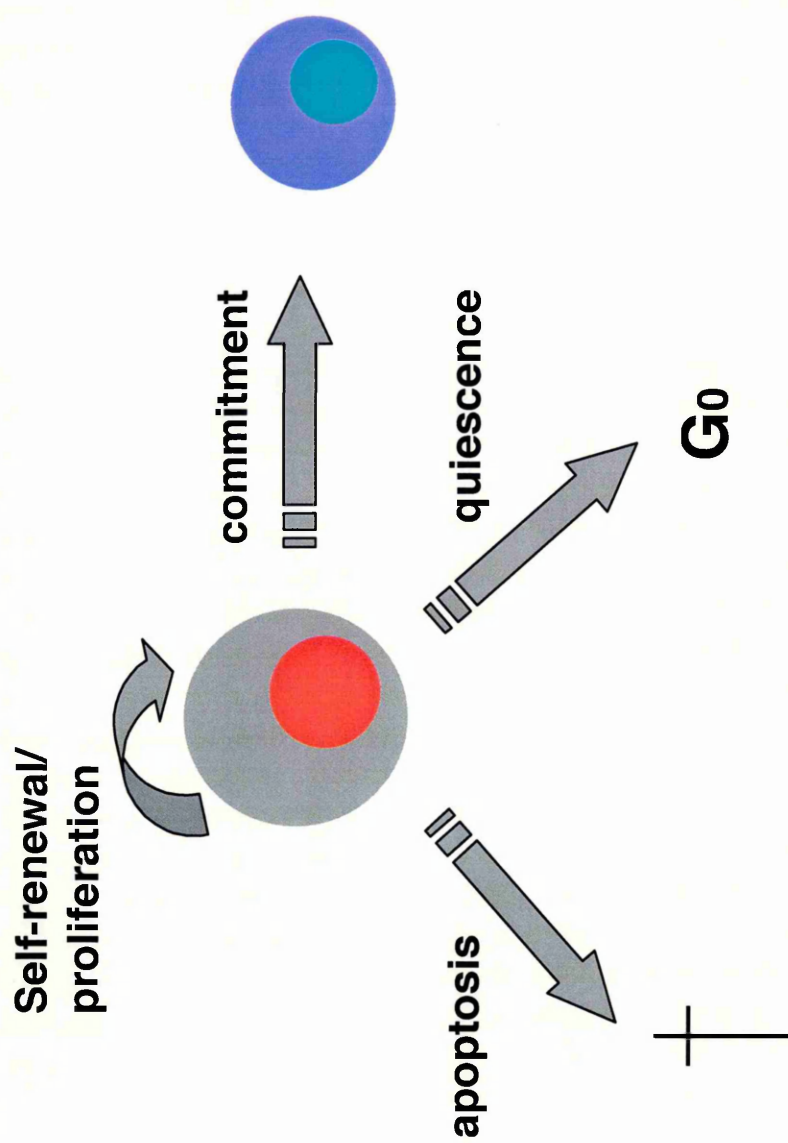


Figure 1.3: Cell fate outcome in haematopoiesis

stochastic and the instructive hypothesis. The stochastic model implies that lineage choice is a cell-intrinsic process that occurs at random whereas the instructive hypothesis stresses the importance of external signals from the bone marrow environment or haematopoietic niche for lineage choice of the HSC. Therefore, one of the central questions of ancient and modern philosophy, namely the role of randomness and determination for the direction of life has entered the haematopoietic field⁵⁰.

Low levels of lineage specific gene expression have now clearly been demonstrated in purified stem cells⁵¹⁻⁵³ suggesting the concept of multilineage priming. This has led to the hypothesis that loss of multipotency may be a stochastic event characterised by a decrease in both the diversity of molecules found in the cell and in the complexity of cellular dynamics⁵⁴. However, there is also increasing evidence of the importance of the bone marrow microenvironment and cell-to-cell communication for cell fate decision⁵²

Taking into account the experimental evidence, it now seems that fate determination of haematopoietic cells is the result of both stochastic and instructive events occurring within the crosstalk between signaling pathways, cytokine responses and transcription factors⁵⁰.

2.2 Signaling pathways

It has been proposed that mechanisms regulating haematopoietic stem cells elaborate on those that establish morphogenesis during embryonic development. Indeed, several signaling pathways that determine cell fate in model organisms have been shown to expand HSCs. These include Wnt⁵⁵, Notch 1⁵⁶, Sonic Hedgehog⁵⁷ and bone morphogenetic protein (BMP)⁵⁸. Besides, a survey of gene expression in stem cells identified genes involved in cell-cell communication⁵²,

indicating that these cells are responsive to environmental signals. Indeed, haematopoietic cells require constant input from their environment for their survival and in the absence of signals such as c-kit and the JAK-STAT pathway undergo apoptosis⁵⁹. However, these signals do not appear to regulate cell differentiation⁶⁰. It is therefore plausible to postulate that commitment to differentiation occurs spontaneously and that signals from the environment are required to maintain stemness. This is illustrated by the fact that LIF and the STAT-3 pathway⁶¹ or the down-stream acting homeodomain protein Nanog⁶² are required to maintain totipotency of the embryonic stem cells *in vitro*. Finally, as HSCs undergo further lineage restriction and the cells are faced with a two-lineage choice instead of a multilineage option, the extrinsic signals that govern the initial decision between self-renewal and commitment to differentiation are recycled for other functions. This is exemplified by the decisive role of Notch1 at several early and late branch points of lymphoid lineage development (for review ⁶³). Taken together, these observations indicate that evolutionary conserved signaling pathways important during embryonic development are reutilized in the adult to regulate HSC numbers and to sustain cell type diversification.

2.3 Cytokines

In addition to these conserved pathways, a network of cytokines only found in vertebrates transmits external signals to the cell. Two of these cytokines, erythropoietin (EPO) and thrombopoietin (TPO), are essential for the production of erythrocytes and megakaryocytes. Interestingly, they share a high degree of homology and both act via the Janus family tyrosine kinase 2 (JAK2) -Signal transducer and activator of transcription (STAT) 5 pathway. Epo-receptors are maximally expressed on erythroid cells at the CFU-E and proerythroblast stage

and the main function of Epo signaling appears to lie in the prevention of apoptosis. TPO on the other hand acts via its receptor c-mpl on expansion of megakaryocyte precursors and terminal differentiation. The debate over the role of cytokines in cell fate determination has focused on the question whether they are permissive or instructive, similar to the question of stochastic versus deterministic mechanisms of lineage choice. Several lines of evidence suggest a permissive role for cytokines in allowing the survival of cells that are already committed to one fate. This is best exemplified by the fact that erythroid colony formation is observed in EPO receptor deficient cells that are stimulated via other types of receptors⁶⁴, suggesting that erythroid cell differentiation is intrinsically determined and that EPO is required only to sustain cell survival. However, several approaches also provide evidence in support of a more active role of cytokines in cell differentiation. For example, thrombopoietin (TPO), was found to be a specific factor that controls megakaryocytic cell proliferation and maturation^{65,66}. Abrogated expression of its receptor Mpl in transgenic mice results in thrombocytopenia and an 85% decrease in the number of megakaryocytes in the bone marrow⁶⁷. Moreover, GM-CSF is required for the generation of granulocytes and macrophages *in vitro* and skews the output of multipotent cells towards these lineages⁶⁰. Besides, it seems that cytokines can also regulate the expression levels of transcription factors, ie G-CSF induces granulocyte differentiation through up-regulation of C/EBP ϵ and C/EBP α , thus altering ratios between C/EBP α and another transcription factor called PU.1 essential for myeloid development⁶⁸. In addition, c-kit signaling downregulates the E12 transcription factor and prevents progression of the B lineage at the pro-B to pre-B transition. This indicates that cytokines can alter transcription factor dosage, thereby shifting the transcription network towards a

particular path, and thus supporting a more instructive role for cytokines in regulating haematopoietic cell differentiation.

2.4 Transcriptional control of haematopoiesis

The molecular machinery responsible for controlling transcription by RNA polymerase II (RNA pol II) is considerably more complex than previously anticipated. Over twenty years of transcription biochemistry and genetics have identified a battery of proteins that aid or abate RNA pol II access to specific regulatory DNA sites, the promoters. The first of these regulatory proteins to be discovered were the sequence-specific DNA binding transcriptional factors. Next to be characterised were the general transcription factors, a set of highly conserved proteins responsible for escorting RNA pol II to the promoter. The more recent regulators to be characterised are the co-activators, co-repressors and chromatin remodelling complexes⁶⁹.

In the ensuing section, I wish to delineate our current understanding of how sequence-specific haematopoietic transcription factors and their co-factors direct the differentiation of the HSC into erythroid, megakaryocyte and lymphoid lineages. Our current understanding of the role of sequence specific transcription factors in the establishment of the haematopoietic hierarchy is summarised in figure 1.4.

2.4.1 Sequence-specific transcription factors

Clearly, one of the key elements in the process of haematopoietic differentiation and progressive lineage restriction is the control of gene expression via sequence-specific transcription factors. These proteins are multimodular and belong to different families. Expression of these factors can be ubiquitous or lineage-specific and binding to DNA is mediated directly or indirectly via specific

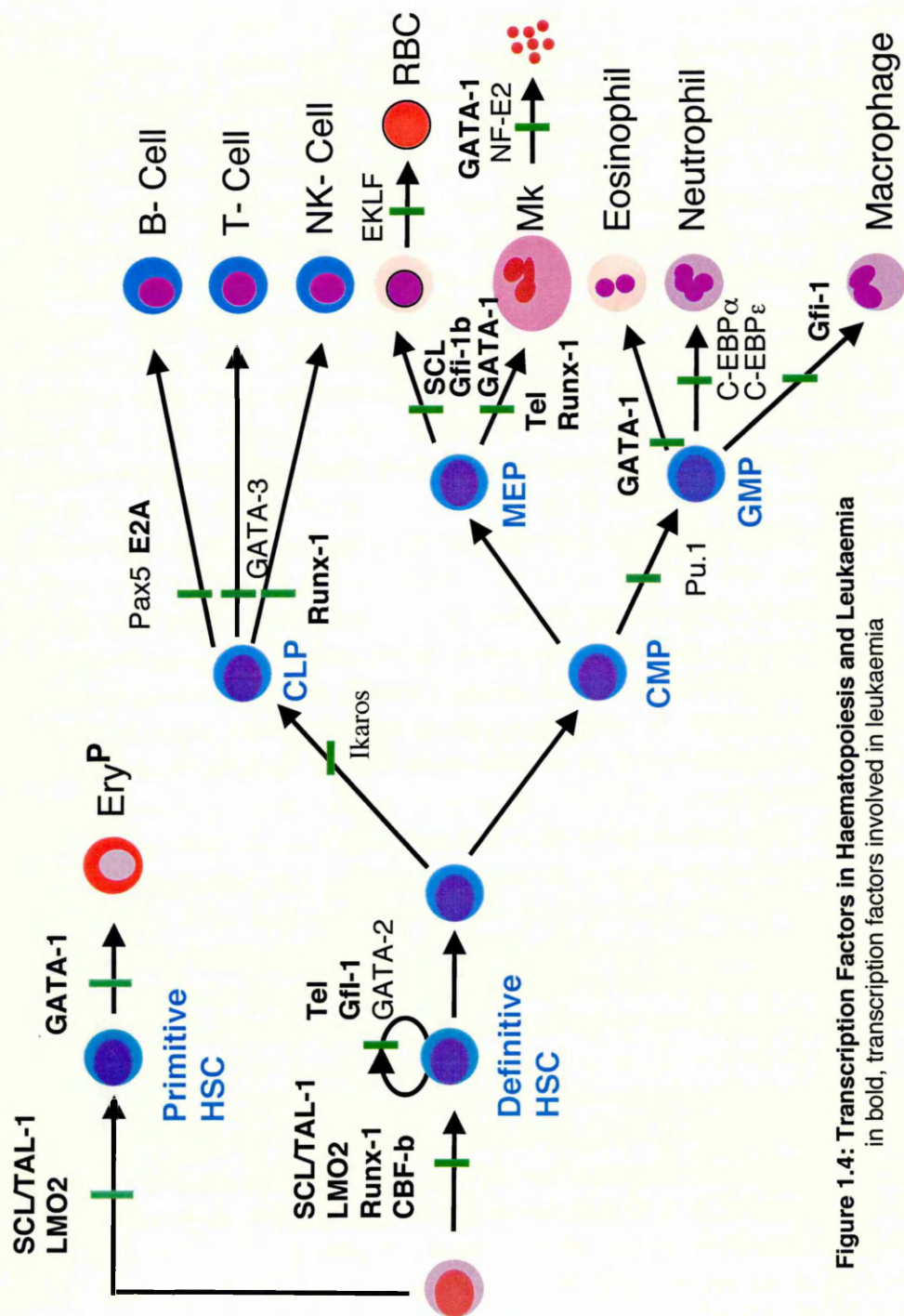


Figure 1.4: Transcription Factors in Haematopoiesis and Leukaemia
in bold, transcription factors involved in leukaemia

DNA sequences. Interestingly, with the exception of GATA-2, all key regulators of haematopoietic differentiation are involved in the pathogenesis of leukaemia.

Haematopoietic transcription factors have been discovered through promoter studies (ie. GATA-1^{70,71}, NF-E2⁷²), because of their involvement in leukaemias (see below) or, more recently, through genetic screens in *Drosophila*, *C. elegans* or zebrafish.

As most of transcription factors important for haematopoiesis in mammals have orthologues in lower vertebrates, these genetic studies have been very informative. As an example, more than 50 mutants with defective haematopoiesis were isolated from large scale mutagenesis screens in zebrafish^{73,74}.

For some mutants, the underlying mechanisms producing the phenotype remain unclear. This is illustrated by the zebrafish *cloche* mutant which was first discovered as a spontaneous mutation in fish from an Indonesian fish farm and was named after its bell-shaped heart (*cloche*=french for bell). It is characterised by an almost complete absence of primitive haematopoietic activity in the ICM and the rostral blood islands and cardiac abnormalities. This is thought to be due to a disruption of the specification of haematopoietic and endothelial systems (for review^{3,75}). Clearly, the characterisation of the function of the proteins encoded by *cloche* and other mutants will give invaluable information to dissect the molecular mechanisms involved in haematopoiesis.

On the other hand, some mutants are defined by a specific defect in one single transcription factor gene. For example, the zebrafish loss-of-function mutant *vlad tepes* harbors mutations of the critical haematopoietic regulator GATA-1⁷⁶ and the severely anaemic mutant « *kugelig* » (named after the German word for spherical),

which was identified in a mutagenesis screen in Tübingen, was recently shown to carry a mutation of the *Hox* gene regulator *cdx4*⁷⁷.

Loss-of-function studies in mammals have been of invaluable help in trying to define the role of key transcriptional regulators. Conventional knock-outs have greatly increased our understanding of the transcriptional mechanisms underlying cell fate decisions. Thus, analyses of mouse knock-out models have identified some of the lineage-restricted transcriptional regulators that play an active role in the specification and biology of the HSC. More recently, conditional knock-out strategies have overcome the limitations of conventional approaches and allowed the examination of the role of these regulators in more differentiated haematopoietic cells. Thus, it has become apparent that all of them also fulfill important roles later on in the differentiation to specific lineages (figure 1.4).

Specification and biology of HSCs

A paradigm of such a transcription factor involved both in HSC specification, subsequent blood lineage differentiation and leukaemia is the bHLH oncoprotein SCL/TAL-1. As such, SCL is a key player in the specification of the primitive and definitive HSC, but not for the biology and maintenance of HSCs^{78,79}.

Besides SCL, other nuclear proteins, LMO-2 and Runx1/AML-1/CbF- β have been shown to play pivotal roles in the early stages of haematopoietic development. LMO-2 inactivation results in failure of erythropoiesis at the yolk sac stage⁸⁰ as well as in a block in definitive haematopoiesis in the adult⁸¹, whereas Runx-1 is absolutely required for the development of definitive haematopoiesis at the fetal liver stage⁸². However, it is no longer indispensable once haematopoietic stem cells are formed⁸³. In addition, the GATA family of zinc finger proteins plays a critical role in haematopoietic development. Loss of GATA-2 offers a clear example

of a factor crucial for the maintenance and proliferation of the HSC, and GATA-2 null cells exhibit a broad haematopoietic deficit⁸⁴. Gfi-1 on the other hand, restricts proliferation and preserves functional integrity of haematopoietic stem cells in competitive repopulation and serial transplant assays⁸⁵. Besides, the Ets transcription factor Tel/ETV6 has recently been identified as the first transcriptional regulator that is selectively required for the survival of adult HSCs through a conditional knock-out strategy⁸⁶.

Progenitor cells

In addition to their role at the HSC level, all transcription factors or their homologues mentioned above are also implicated in the differentiation of haematopoietic progenitors.

SCL, for example, is required for the differentiation of erythroid progenitors and megakaryocytes at the colony forming unit stage⁸⁷⁻⁸⁹. Loss of GATA-1 impedes survival of erythroid progenitors as well as terminal differentiation of erythroid cells, although the consequences for definitive cells are blunted by partial redundancy with GATA-2⁹⁰. GATA-1 is essential for primitive and definitive erythropoiesis and GATA-1 deficient mice die by 12.5dpc due to inefficient erythropoiesis in the yolk sac⁹¹. Besides, *in vitro* and chimeric analyses of the knock-out^{92,93} and knock down^{94,95} models of GATA-1 resulted in arrested embryonic erythropoiesis and a maturation arrest of definitive haematopoiesis at the proerythroblast stage. Loss of GATA-1 expression in megakaryocytes also leads to defects in maturation in this lineage^{96,97}. Likewise, mice missing the lymphoid factor Ikaros lack T- or B lymphoid cells, and their precursors⁹⁸. PU.1-null mice lack cells of disparate haematopoietic lineages, namely myeloid and lymphoid cells⁹⁹. More surprisingly, loss of some broadly expressed factors results in lineage-selective deficits. For example,

targeted disruption of the E2A gene blocks B-lymphopoiesis, but does not interfere with development of other tissues in which it is normally expressed^{100,101}.

Terminal differentiation

Conversely, some cell-restricted transcription factors appear to be required only after lineage commitment. For example, in terminal myeloid differentiation, Gfi-1 is required for neutrophil differentiation¹⁰² and the CCAAT/enhancer-binding-protein- α (C/EBP- α) coordinates cellular differentiation with growth arrest in neutrophils¹⁰³. In erythro-megakaryocytic adult hematopoiesis, loss of p45 NF-E2 and FOG-1 expression impairs maturation of both the erythroid and megakaryocytic lineages¹⁰⁴⁻¹⁰⁶, and EKLF deficiency produces a stage-specific, gene selective defect : failure of adequate transcription of the adult β globin gene¹⁰⁷.

Taken together, these data illustrate that a complex interplay between external signaling pathways and transcription factors gives rise to the progressive lineage restriction and differentiation of the HSC and establishes the hierarchy of all blood cell lineages including the erythroid (figure 1.5) and megakaryocyte (figure 1.6) compartment.

A Stage of erythroid differentiation



B Surface marker expression

CD71+ Ter119+

C Transcription factors

SCL
LMO-2
GATA-2
GATA-1
FOG
RB
EKLf
NF-E2

D Receptors for required haematopoietic growth factors

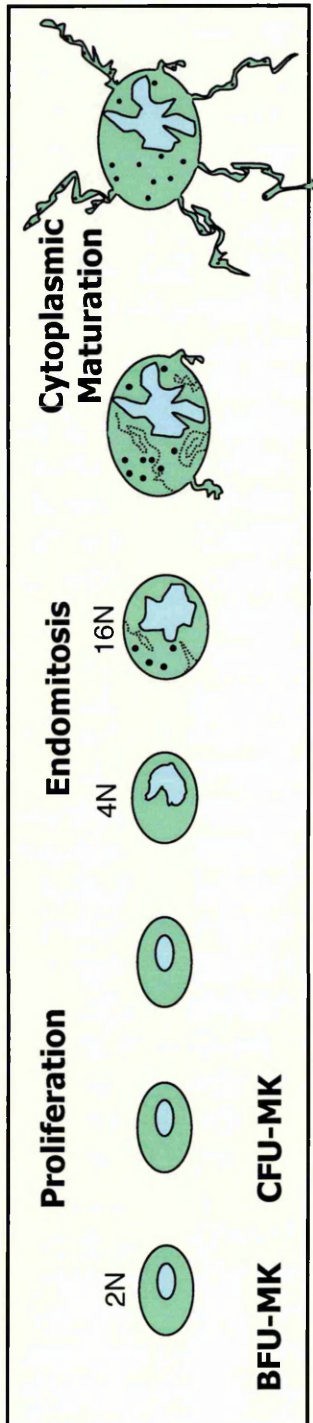
c-kit
EPO-R
IGF-1-R

E Globin expression

Adapted from Koury et al, 2002⁴²

Figure 1.5: Erythroid development

A Stages of megakaryocyte differentiation



B Surface marker expression _____ GPIIbGPIIIa

_____ PF4, vWF

C Transcription factors

_____ SCL

_____ LMO-2

_____ GATA-2

_____ GATA-1

_____ FOG

_____ Ets family members

_____ P 45 NF-E2

D Receptors for required haematopoietic growth factors

_____ c-mpl

_____ c-kit

_____ IL-3R

_____ GM-CSFR

_____ IL-6

_____ IL-11

_____ LIF

Figure 1.6: Megakaryocyte development

2.4.2 Leukaemogenic transcription factors

As mentioned above, many of these transcription factors were originally cloned from chromosomal translocations in human leukaemia. Indeed, transcription factor genes constitute the most frequent target of leukaemic translocations, emphasizing the critical role of these « master » regulatory proteins in the control of blood cell development¹⁰⁸. Activation of transcription factor genes by chromosomal translocations takes two main forms. In T- or B- cell progenitors, such genes are frequently mobilised into the vicinity of genes encoding discrete chains of the T-cell receptor (TCR) or immunoglobulin (Ig) molecules, resulting in inappropriate expression of the translocated proto-oncogenes. More commonly, the coding exons of genes disrupted by a reciprocal translocation are incorporated into a single fusion gene, which generates a chimeric protein with unique properties including loss-of-function or dominant negative alterations.

Translocations that inappropriately activate transcription factor genes in acute lymphoblastic leukaemia and acute myeloblastic leukaemia show remarkable specificity for haematopoietic cells blocked at defined stages of differentiation. This property suggests that the different oncoproteins produced by these chromosomal changes specifically interfere with the transcriptional networks that normally function in concert with growth factors and their receptors to regulate haematopoiesis¹⁰⁹. The progression to leukaemia then depends on additional mutations that promote the survival of developmentally arrested cells (two-hit theory).

2.4.3 Mechanisms of action

In recent years, it has become clear that the principles governing the transcription factor network are far more complex than previously thought.

Dosage

One of the most important realisations was that transcription factor dosage plays a central role in the determination of cell fate as exemplified by the E2A gradient in lymphoid differentiation^{110,111} and the PU.1 dosage effects regulating B cell versus macrophage development¹¹².

Protein-protein interactions : antagonism versus cooperation

Transcription factors commonly exert their function via protein-protein interactions. In this molecular crosstalk, different nuclear proteins can both antagonise each other or cooperate together in multimeric protein complexes.

As exemplified by the GATA-1/PU.1 interaction, specific transcription factors may be able to antagonise each other by inactivating DNA-binding domains¹¹³ or by blocking protein interaction domains¹¹⁴ thereby determining cell fate.

Besides, these factors cooperate as part of multimeric large molecular weight protein complexes with activating or repressing functions. The molecular mechanisms by which the same sequence-specific transcription factor can activate or repress gene expression are poorly understood, but it seems that co-factor exchange is commonly used to regulate levels of transcriptional activation. Thus, it has been shown that different classes of transcription factors can recruit both co-repressors and co-activators^{115,116} as recently exemplified by NF-E2p18/MafK whose dimerisation partner switches from co-repressor to co-activator during MEL cell differentiation¹¹⁷. These data illustrate the importance of protein-protein interactions between transcription factors and their co-factors for haematopoietic

cell fate determination. They also suggest that the classical view of haematopoietic lineage determination via positive activation of unique gene expression programs is too simplistic. Instead, it seems that lineage-specific transcription factors simultaneously exert inhibitory effects on alternate lineage gene programs by either directly antagonizing other transcription factors or by recruiting inhibitors of transcription to large multimeric complexes.

As illustrated in the next paragraph, the mechanism through which co-factors of sequence specific transcription factors confer activation or repression frequently involves epigenetic processes such as histone modifications or DNA methylation.

2.4.4 Transcription co-factors and the context of chromatin

A general and very much simplified schematic overview of the mechanisms surrounding the chromatin modifications involved in directing gene expression is shown in figure 1.7 (for review¹¹⁸⁻¹²¹).

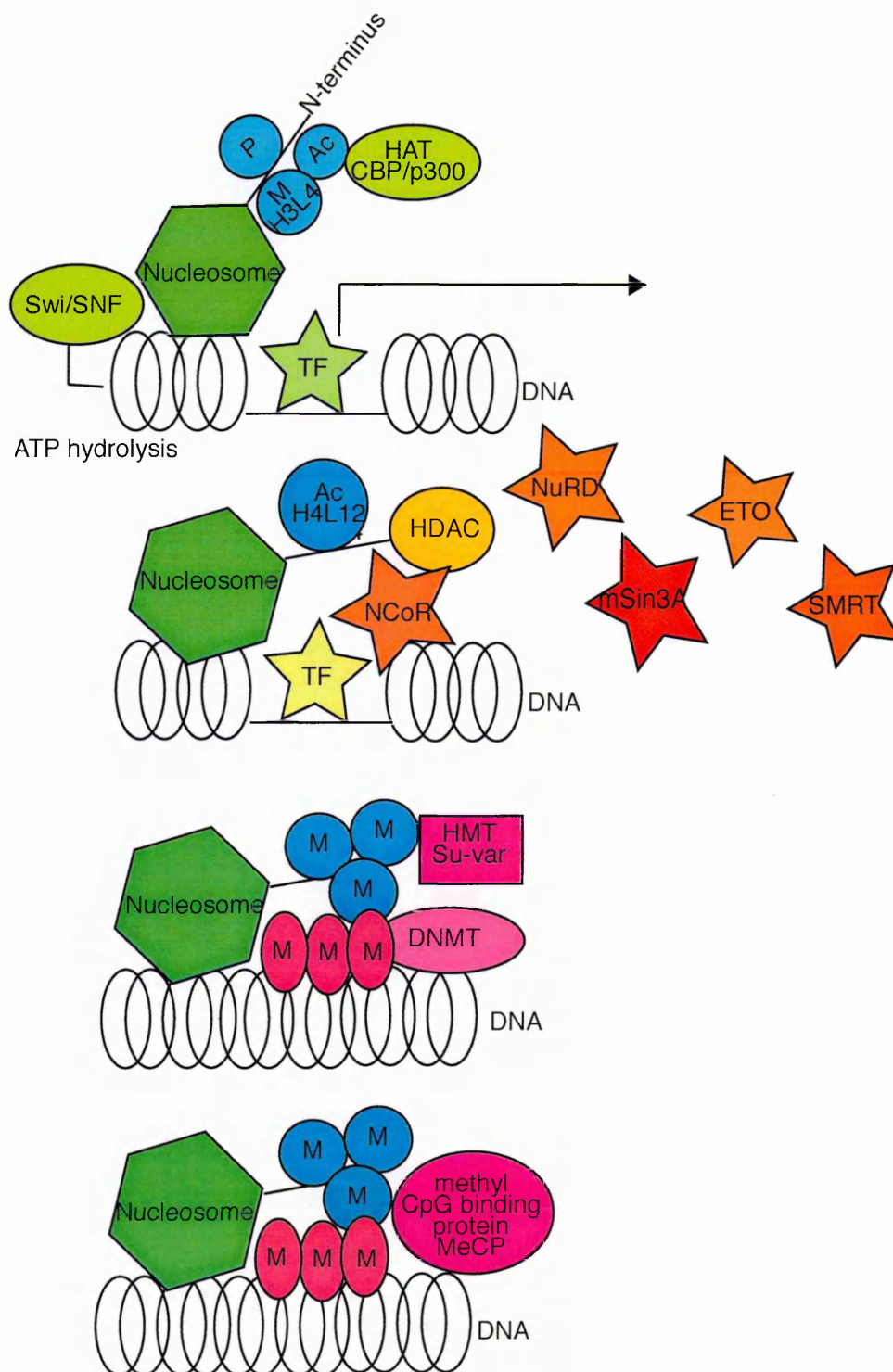
The eukaryotic genome is packaged into the compact state of chromatin that forms the scaffold from which the fundamental nuclear processes of transcription, replication and DNA repair occur. Chromatin is composed of nucleosomes that are comprised of 145-147 base pairs of DNA wrapped around an octameric core containing two molecules each of histones H2A, H2B, H3 and H4. The H1 linker stabilizes the assembly of the octameric core into higher order structures. The N-terminal portion of the core histones is the site for post-translational modifications such as methylation, phosphorylation, ubiquitylation and acetylation. These post-translational histone modifications have been implicated in replicational and transcriptional activity¹²². Chromatin remodeling via ATP-dependent hydrolyses of the Swi/SNF family of co-activators (for review¹²³) and modifications by histone acetylases (HAT, like p300/CBP, PCAF) or histone deacetylases (HDAC)

represent a fundamental mechanism of transcriptional regulation. It has been proposed that histone acetylation or specific methylation at histone 3 lysine 4 weakens the interactions of histones with DNA and induces alteration in nucleosome structure, enhancing accessibility of targeted promoters to components of the transcriptional machinery¹²⁴.

Conversely, decreased histone acetylation due to the action of HDACs or acetylation at histone 4 lysine 12 is thought to lead to less accessible chromatin conformation, resulting in repression of transcription^{125,126}. Unlike activation complexes, in which co-activators have intrinsic HAT activity, one of the functions of co-repressors, including N-CoR¹²⁷, SMRT¹²⁸ and mSin3A¹²⁹, is it to recruit HDACs to large multi-protein repression complexes. These complexes also contain sequence specific transcription factors and other molecules such as the ETO family of co-repressor proteins^{130,131}.

Deacetylation might then prompt methylation of histone tails by histone methyl transferases like Su-var leading to condensation of the chromatin structure and subsequent recruitment of DNA methyl transferases. Methylated DNA is bound by methyl CpG binding proteins such as MeCP and HP1, which induces higher order formation of heterochromatin. At this stage, the DNA is compacted and inaccessible. However, to which extent this degree of heterochromatin formation is irreversible remains unknown.

In summary, it now seems plausible that, analogous and complementary to the genetic code identified 40 years ago, the different histone and chromatin modifications described above, encode for a « histone code », that further defines transcriptional regulation¹¹⁸.



modified from Melnick et al, 2002¹²⁰,
Jenuwein et al, 2002¹¹⁸ and Zhang et al, 2001¹²¹

Figure 1.7 Summary of histone modifications and co-repressors

See main text for explanations.

P=phosphorylation; M=methylation; Ac=acetylation; HAT=histone acetyl transferase; TF=transcription factor; HDAC= histone deacetylase; HMT=histone methyl transferase; DNMT=DNA methyl transferase

2.4.5 The role of co-repressors in leukaemia

Inappropriate repression of genes required for cell differentiation has been linked to several forms of cancer, particularly to acute leukaemia. For example, in acute promyelocytic leukaemia (APL), the retinoic acid receptor (RAR) fusion proteins (e.g. PML-RAR or PLZF-RAR) can interact with components of a co-repressor complex containing HDACs¹³². Additionally, it has now become apparent that the fusion protein AML1/ETO acts in an analogous way by recruiting HDAC containing repressor complexes to AML-1 DNA binding sites, thereby inappropriately inactivating genes important in haematopoietic differentiation^{130,131,133,134}. Therefore, molecules inhibiting the activity of HDACs have gained much interest as possible new therapeutic agents in the management of acute leukaemia¹³⁵⁻¹³⁸. More recently, it has been shown that expression of the AML/ETO fusion protein (or ETO on its own) in haematopoietic cells impedes binding of the co-activator p300 to E2A¹³⁹, suggesting that repression of gene activation might be mediated by blocking the interaction between co-activator and transcription factor. However, the exact role of ETO proteins in normal and malignant haematopoiesis remains to be elucidated.

3 THE ONCOPROTEIN AND bHLH TRANSCRIPTION FACTOR SCL/TAL-1

3.1 History of discovery

The story begins with a case report of a 16-year old boy who presented with acute lymphoblastic leukaemia (ALL) of an early T-cell phenotype and resistant to

standard therapeutic agents¹⁴⁰. Surprisingly, after receiving the adenosine deaminase inhibitor 2'deoxycoformycin, the leukaemic cells underwent transformation into myeloid cells. Unfortunately, the patient died due to extensive tissue infiltration with cells of a promyelocyte morphology. Both before and after the transformation, leukaemic blasts carried a translocation between chromosome 1p32-33 and the T-cell receptor locus on chromosome 14q11. The investigators established a cell line called DU.528 carrying the same cytogenetic abnormality and recapitulating the stem cell phenotype in vitro¹⁴¹. However, since then, there have been no further reports that leukaemias carrying this type of translocation behaved in a similar way after treatment. When the breakpoint was isolated, it became apparent that as a result of the translocation a new gene on chromosome 1 was abberantly activated under the control of the TCR locus. The gene was cloned independently by several groups and given the name STEM CELL LEUKAEMIA (SCL, also called Tal-1 and Tcl5)¹⁴²⁻¹⁴⁶. Although we now know that abberant expression of SCL leads to leukaemia of the early T cell type and not to stem cell leukaemia, this name remains very appropriate, given SCL's pivotal function in fate determination of the early haematopoietic stem cell.

3.2 Expression profile

During embryogenesis, SCL is first expressed in the presumptive haemangioblast¹⁴⁷. Then, SCL is present in the HSC and the most immature progenitors, but becomes downregulated as differentiation proceeds into most lineages^{40,148-151}. However, SCL remains expressed in erythroid cells, megakaryocytes and mast cells¹⁵²⁻¹⁵⁶.

Outside the haematopoietic system, SCL expression has been observed in endothelial and neurogenic tissues¹⁵⁷⁻¹⁵⁹.

3.3 Regulation of *scf* gene expression

Two promoters in alternative 5' exons have been identified in both the human and murine *scf* gene^{160,161} together with a third promoter within the gene whose function remains to be elucidated¹⁶². The two 5' promoters exhibit lineage restricted activity. Promoter 1a is regulated by GATA-1, SP1 and SP3 and is active in transient reporter assays in erythroid and mast cells, but not in T-cells. Promoter 1b is regulated by PU-1, SP1 and SP3, and is active in mast cells and early CD34+ myeloid precursors¹⁶³⁻¹⁶⁶. In addition, multiple enhancer elements have been identified allowing expression of the *scf* gene in a chromatin context¹⁶⁷⁻¹⁷⁰. Thus, reporter assays in transgenic mice have demonstrated a role for the upstream enhancers in directing *scf* gene expression to neurogenic and endothelial tissue whereas the downstream enhancer elements targeted *scf* gene expression to the haematopoietic and endothelial systems¹⁷¹⁻¹⁷³. Furthermore, it was shown in rescue experiments that stem cell specific enhancer elements are able to re-establish the formation of haematopoietic progenitors in a *scf*^{-/-} background^{174,175}. In the same experimental approach, the *scf*+18/19 stem cell enhancer directs transcription to both haematopoietic precursors and endothelial cells through binding of Fli-1 and Ets factors¹⁷⁰.

In view of the discordant protein and mRNA levels of SCL in erythroid differentiation, it has been postulated that other mechanisms in addition to these *cis*-regulatory elements must be responsible for fine tuning of protein turn over¹⁷⁶, although these post-transcriptional regulatory events remain to be uncovered.

3.4 Structure

SCL is a member of the bHLH family of transcription factors

These transcription factors share a common basic helix-loop-helix (bHLH) domain which mediates sequence-specific interaction with DNA through the basic domain^{177,178} and promotes homo- or heterodimerization and protein-protein interaction through the HLH domain^{179,180}. bHLH proteins have been subgrouped into seven different classes¹⁸¹. Of these, class I includes the ubiquitously expressed E proteins represented by the *E2A* gene products E12/47, HEB and E2-2. All members of this group share a significant homology in their two transactivation domains and are conserved throughout evolution.

Class II bHLH proteins are tissue specific and include MyoD¹⁸² or Neuro D¹⁸³. Class V members are represented by the Id proteins. They lack the DNA binding domain and act as dominant-negative regulators of class I and class II proteins¹⁸⁴.

SCL belongs to the family of class II bHLH transcription factors and heterodimerizes with the class I bHLH proteins E2A^{181,185,186}. These heterodimers recognize a specific nucleotide motif, CANNTG, termed the E-box and were initially thought to function in transcriptional regulation via direct DNA-binding.

Core region : bHLH domain

Although important domains have been mapped to the Nt and Ct regions of SCL in transactivation assays in heterologous cells^{167,187}, a structure-function study performed in SCL-null ES cells has shown that the bHLH domain is the core region of the protein. This study convincingly demonstrated that a construct containing the minimal bHLH region of SCL was fully active in generating primitive and definitive haematopoiesis¹⁸⁸.

« *To bind or not to bind DNA* »

The classical view that transcription factors act via direct binding to DNA has recently been challenged by the observation that transcription factors act in the context of multi-subunit protein complexes and do not necessarily require a DNA-binding domain to exert their function. The structure-function study of SCL has given further proof that transcription factor function can be divided into DNA-binding dependent and independent actions.

In this context, it has been shown in SCL^{-/-} ES cells and in the zebrafish mutant *cloche* (see chapter 2.4.1) that DNA-binding defective mutants are able to specify HSC and restore primitive haematopoiesis. However, these mutants were unable to rescue maturation of definitive erythroid cells and megakaryocytes¹⁸⁸. In accordance to this data, at a molecular level, Lecuyer et al demonstrated that direct DNA-binding of SCL on the *c-kit* promoter is not necessary for activation of *c-kit* expression in a progenitor cell line¹⁸⁹, whereas maximal activation of the *GPA* promoter in mature erythroid cells requires SCL DNA-binding¹⁹⁰.

As far as T-ALL is concerned, two mouse models have now convincingly shown that the DNA-binding activity of SCL is not required to reproduce a preleukaemic or leukaemic phenotype^{191,192}.

Therefore, it is likely, that, to specify HSC and in leukaemogenesis (as will be described below), SCL acts through its HLH domain by protein-protein interaction.

The HLH region

In order to define residues within the SCL HLH domain necessary for the haematopoietic function of SCL, a detailed mutational analysis of this region was performed¹⁹³. This study showed that most of the amino acids in the loop and a

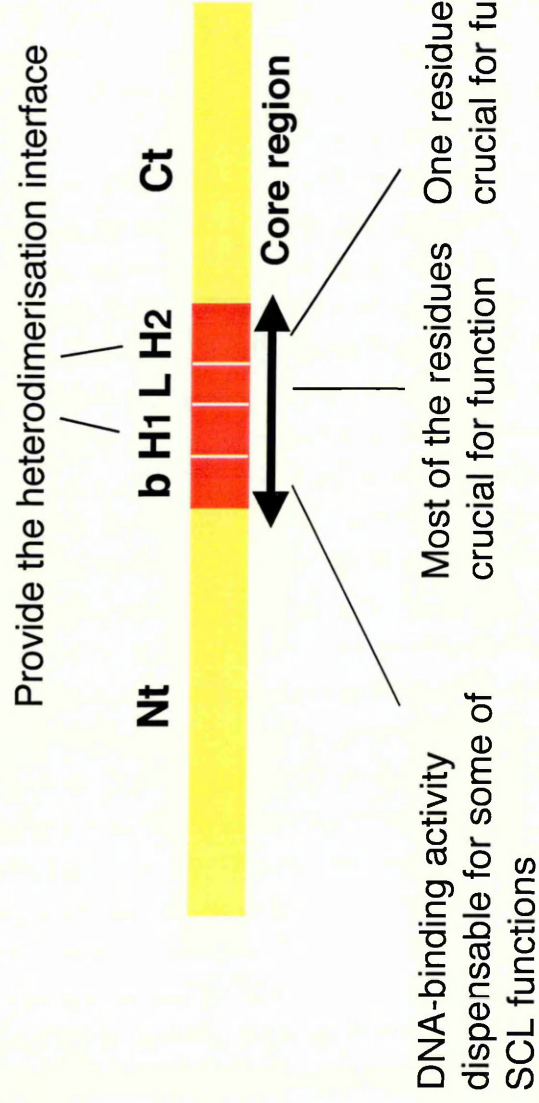
phenylalanine residue in helix two are crucial for SCL function in haematopoietic specification (summarised in figure 1.8). Importantly, introduction of these residues is sufficient to convert the muscle-specific HLH domain of Myo-D into one capable of rescuing haematopoiesis from SCL^{-/-} ES cells. It is very likely that some if not all the residues thus defined mediate protein-protein interactions.

Post-translational modifications

Post-translational modifications of SCL influence both its function as a regulator of erythroid-megakaryocytic differentiation and its DNA-binding affinity.

As a result of multiple transcription initiation sites within the *scf* gene, the SCL protein exists in several different forms containing different N-terminal portions of the full length protein while retaining the bHLH domain^{142,160,161}. The distribution of these SCL isoforms is cell type specific, suggesting that they may influence cell fate depending on their ability to transactivate downstream target genes¹⁹⁴. In this context, it has recently been demonstrated that SCL isoform production is controlled by a small open reading frame which is responsive to signaling pathways that modulate translation initiation factors. This study concluded that different isoforms may direct differentiation towards the erythroid or megakaryocytic lineages¹⁹⁵.

Further posttranslational modifications include phosphorylation of serine residues and acetylation. Thus, phosphorylation of serine residues^{196,197} can influence the DNA-binding activity of SCL. Acetylation of SCL by the histone acetyltransferases p300/CBP and p/CAF promotes erythroid differentiation^{198,199}.



Porcher et al, 1999¹⁸⁸
Schiaeger et al, 2004¹⁹³

Figure 1.8: Results of the Structure-Function Study of SCL

3.5 SCL function

In normal haematopoiesis, SCL plays crucial roles at different stages of development.

SCL is indispensable for generation of primitive and definitive haematopoietic stem cells

Different approaches convincingly show that SCL is required for the commitment of the haemangioblast to a haematopoietic fate^{148,200-205}. Gain-of-function studies in zebrafish also suggest that SCL can specify the haemangioblast from early mesoderm²⁴ and together with LMO2 induces early blood and endothelial genes all along the anterior-posterior axis²⁰⁶.

Besides, SCL knock-out mice die at 9.5 dpc due to the complete absence of primitive haematopoietic cells, revealing that SCL is absolutely required for primitive haematopoiesis^{87,88}.

Further studies using SCL^{-/-} ES cells, both in *in vitro* differentiation cultures and *in vivo* in chimeric mice, showed that lack of SCL activity also induces a complete absence of all definitive haematopoietic lineages and concluded that SCL was indispensable for the generation of both primitive and definitive blood cells^{89,207}. These findings have been further substantiated by a study using a conditional knock-out strategy, which revealed that although SCL is required for the generation of haematopoietic stem cells, its inactivation does not impair stem cell maintenance or function in serial transplantation experiments⁷⁸. A second study, equally making use of a conditional knock out, but following a different Cre-recombinase induction protocol, found a two-fold reduction in primitive progenitors, such as CFU-S12⁷⁹

and a similar reduction in short term repopulating cells after transplantation whereas the number of long term repopulating stem cells remained the same²⁰⁸.

SCL is a critical regulator of adult erythroid and megakaryocyte differentiation

In addition to its crucial role at the earliest stages of primitive and definitive haematopoiesis, SCL is also thought to act as an important transcription factor for the differentiation of erythroid and megakaryocytes. The first clue to this came from antisense experiments showing that SCL regulates proliferation and self-renewal of the multipotent progenitor cell line K562²⁰⁹. Its ability to promote erythroid differentiation was then investigated by forced expression in induced MEL cells²¹⁰, TF-1 cells¹⁵⁵ and the K562 cell line²¹⁰. In all of these studies, SCL was shown to enhance differentiation into the erythroid lineage. Further over-expression studies of SCL using retroviral transfer into haematopoietic CD34+ progenitor cells have given evidence that SCL might promote erythroid and megakaryocyte differentiation at the expense of myelocyte differentiation^{211,212}. Recently, genetic evidence has emerged clearly demonstrating that SCL is required for the maturation of erythroid and megakaryocytic lineages^{78,79,188}.

In the past few years, it has become obvious that in order to exert these functions in primitive and definitive haematopoiesis, SCL interacts with other nuclear proteins in multiprotein complexes.

3.6 SCL partners

As mentioned above, the tissue specific class II bHLH protein SCL is able to form heterodimers with ubiquitous class I bHLH proteins such as the E2A gene products E12 and E47, HEB and E2.2. In addition, SCL has been shown to interact with other proteins (figure 1.9).

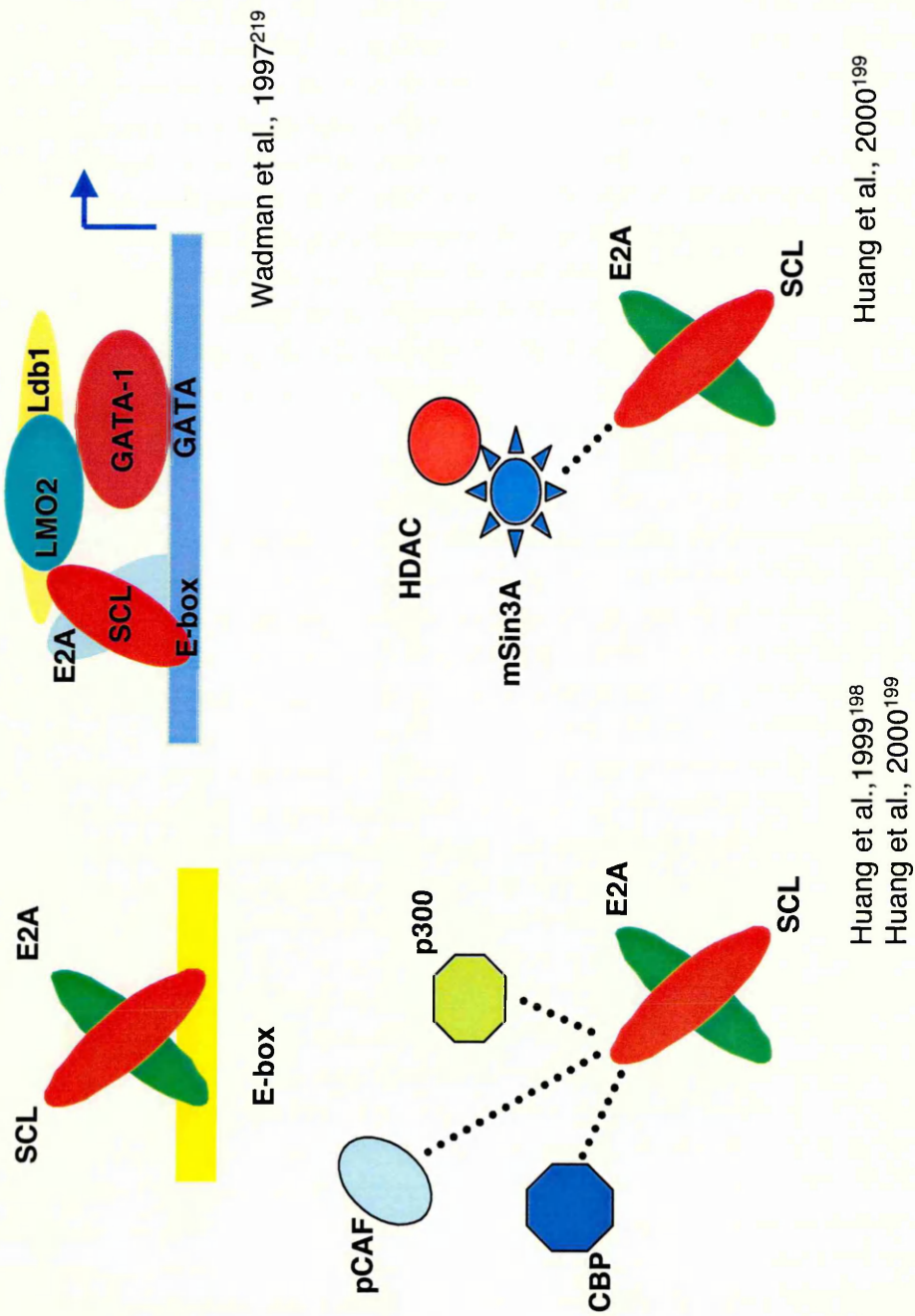


Figure 1.9 Partners of SCL

The first SCL partners to be identified by co-immunoprecipitation in a T-ALL cell line²¹³ or by mammalian two hybrid assay²¹⁴ were the LIM domain proteins LMO-1/2. LMO-1 and LMO-2 share a high degree of homology and, like SCL, have been implicated in the pathogenesis of T-ALL²¹⁵. LMO-2 is also essential for normal blood cell formation^{80,81}. Besides, there is increasing evidence for both physical and functional interaction between bHLH heterodimers, LIM-domain proteins and LIM-domain binding proteins such as LDB-1^{216,217}. Targeted deletion of the mouse *LDB-1* locus causes a severe patterning defect during gastrulation and abnormalities within the mesoderm derived extraembryonic structures including the blood islands of the yolk sac²¹⁸, thus indicating a role for this protein in blood cell formation. Using casting experiments, Wadman et al revealed the presence on DNA of a pentameric complex in induced MEL cells consisting of the SCL/E2A heterodimer together with LMO-2, LDB-1, and the critical haematopoietic regulator GATA-1²¹⁹. Besides, Valge Archer described a similar complex consisting of SCL, LMO-2, LDB-1 and the GATA family member GATA-3 in T-ALL²¹³.

Interestingly, interactions of SCL with the histone acetyl transferases CBP/p300 and pCAF^{198,199} as well as with the co-repressor mSin3A and associated HDACs²²⁰ have been reported.

3.7 SCL target genes

Despite the fact that SCL is an essential regulator of haematopoiesis, only few target genes have been identified so far. Until recently, the evidence for these potential candidates was mostly based on the presence of an E box within their promoter or enhancer regions and the outcome of reporter assays rather than direct evidence for the *in vivo* presence of a protein complex including SCL. In this context, a DNAase hypersensitive site (HS1) upstream of the GATA-1 gene

containing an E-box-GATA motif, has been shown to direct reporter gene expression to erythroid cells and megakaryocytes in transgenic mice²²¹. Similarly, EKLF and Flk-1 genes have been described as potential candidate targets in erythroid and endothelial cells, respectively^{222,223}. Besides, using chromatin immunoselection, SCL was found to bind to the regulatory sequences of a gene of unknown function with homology to ontogelin²²⁴. Among the first direct target genes described for SCL was the *c-kit* gene, which encodes an essential tyrosine kinase receptor required for the maintenance of HSC and progenitors^{189,225}. Using chromatinimmunoprecipitation (ChIP) assays, Lecuyer et al showed that a SCL multiprotein complex consisting of E47, LMO-2, LDB-1, GATA-1/2 and Sp1 can assemble on the *c-kit* promoter and that the presence of all these proteins was necessary for transactivation of the gene in a pluripotent CD 34+ cell line. However, *c-kit* gene expression was not affected in SCL-/- ES cells induced to undergo haematopoietic differentiation¹⁴⁸. In addition, Vitelli et al demonstrated that retinoblastoma protein can associate with SCL, E12, LMO-2 and LDB-1 in more mature erythroid cells and leads to repression of *c-kit* expression²²⁶. Finally, the pentameric complex including SCL is believed to positively regulate expression of late erythroid genes such as glycophorin A (GPA) and protein 4.2 genes^{190,227}.

3.8 SCL: activator and repressor

Functionally, SCL is considered as both a positive and a negative regulator. First, because SCL/E2A heterodimers are less potent activators than E2A homodimers, it was postulated that SCL could exert repressor effects^{228,229}. Second, as mentioned above, SCL positively regulates erythroid differentiation when overexpressed in MEL cells²¹⁰, but is a repressor of erythroid differentiation of the human cell line K562 in antisense experiments²⁰⁹ and blocks differentiation when

ectopically expressed in normal and leukemic myeloid precursors²³⁰. Finally and as indicated before, the pentameric complex is believed to positively regulate expression of glycophorin A (GPA) and protein 4.2^{190,227}, and to activate or repress expression of the *c-kit* gene depending on the cellular context and nature of additional partners^{189,226}. In addition to their functional activity observed in transcriptional assays, members of the pentameric complex have been shown to co-occupy *in vivo* regulatory sequences of GATA-1, α -globin and GPA genes^{190,231,232}. Although we are now beginning to understand how a haematopoietic-specific multiprotein transcriptional complex might form on regulatory regions of tissue-specific genes, so far, the composition of the complex does not account for its function. Activation and repression of gene expression are achieved through multiple mechanisms including recruitment of chromatin remodelling factors and histone-modifying proteins like acetyltransferases (HATs) and deacetylasetransferases (HDACs). As mentioned above, interaction of SCL with HATs CBP/p300 and pCAF^{198,199} as well as with the co-repressor mSin3A and associated HDACs²²⁰ have been reported, thereby reinforcing the hypothesis that SCL might have a dual function, as an activator or a repressor.

3.9 SCL and T-cell leukaemogenesis

The t(1;14) translocation is the most frequently observed chromosomal rearrangement in acute T-cell leukaemia and leads to overexpression of SCL in early T-cells, where it is normally downregulated^{144,146,162,233,234}. Ectopic expression of SCL also occurs without obvious chromosomal rearrangement, but due to a deletion of about 90kb of DNA from chromosome 1 which brings the *scf* gene under the control of the ubiquitously activated *si* promoter, thus leading to constitutive expression of SCL²³⁵⁻²³⁹. This deletion is believed to result from

illegitimate recombinase activity. Recently, a third mechanism of SCL RNA overexpression in T-ALL has become apparent involving the biallelic expression of SCL^{240,241}, but the underlying molecular mechanisms and their impact on protein levels remain to be elucidated. The incidence of these different abnormalities was defined by gene expression data from microarray and quantitative RT-PCR on 59 children with T-ALL and normal thymocyte controls showing that within this population SCL over-expression occurred in 49% of all cases. None of the 29 SCL positive samples carried the t(1;14). Intrachromosomal deletions occurred in 31 % of case. In the remaining 69%, no cytogenetic or molecular evidence of rearrangements affecting the SCL locus could be detected²⁴².

The oncogenic properties of SCL have been studied using transgenic mouse models with a variety of different promoters and mouse backgrounds^{191,243-246}. It is now clear that the oncogenicity of SCL alone in these mouse models is weak. Dependent on the mouse strain and the promoter used, mice either failed to suffer from leukaemia or developed the disease only after a long latency period. This led to the conclusion that either levels and/or the precise timing of SCL expression in T-cell development may be critical for leukaemogenesis. Moreover, additional oncogenic events are required to enhance the leukaemic potential of SCL. One potential candidate partner of SCL in this setting could be the LIM domain proteins LMO-1/2, which are also involved in human T-cell leukaemogenesis²⁴⁷. Moreover, microarray data on T-ALL samples confirmed that LMO-1/2 are over-expressed together with SCL in a high proportion of cases²⁴² and, using biochemical assays, Wadman et al showed that LMO-1/2 and SCL can associate in extracts of leukaemic blasts²¹⁴. In addition, transgenic mouse models expressing both LMO-

1/2 and SCL in thymocytes develop aggressive leukaemia and T-cell lymphoma with low latency^{244,245,248,249}.

The LIM proteins are unable to bind to DNA and the LIM domain of LMO-2 is regarded as a protein-protein interaction domain. This implies that LMO proteins may serve as adapters allowing the proper assembly of larger multi-protein complexes^{250,251}. Hence, LMO-2 and SCL together with E47 and LDB-1 were found in an aberrant complex in a leukaemic T cell line²⁵². These aberrant complexes might interfere with normal differentiation of T cells by deregulating gene expression through multiple possible ways. First, SCL-containing complexes could lead to abnormal gene activation. Thus, when expressed in a SCL/LMO2-negative T-ALL cell line, SCL or its DNA binding mutant, and LMO-2 form a complex with GATA-3 and specifically induce expression of the TALLA-1 and RALDH2 genes^{253,254}, although the relevance of the expression of these genes for the leukemogenic process remains to be clarified. Second, SCL heterodimerisation partners, the E-proteins, are essential regulators of T-cell differentiation and their inactivation leads to T-cell leukaemia^{111,255}. It has been long hypothesised that SCL might prevent the function of E-proteins in T cells through simple sequestration^{149,228,229}. In addition to sequestering E proteins, SCL could recruit repressor complexes to genes normally switched on in differentiating T cells. Two papers now suggest that both these mechanisms may be operative. Herblot et al demonstrated that in mice, SCL and LMO-2 are normally expressed in the double negative thymocyte population, but progressively downregulated up to the CD4+ CD8+ stage. However, in the leukaemic setting, SCL is over-expressed in the late double negative and in the double positive stage. SCL and LMO-2 over-expression in mice leads to inhibition of E2A/HEB function and repression of the pre-Ta gene

expression, essential for normal T cell development¹⁴⁹. Consistent with this, O'Neil et al showed that expression of a SCL transgene in an E2A or HEB heterozygous background promotes development of leukaemia. Using ChIP assays, they demonstrated that the SCL/E2A heterodimer recruits the co-repressor complex mSin3A/HDAC1 to enhancer elements of genes important for thymocyte differentiation, such as CD4 and pre-T α ²⁵⁶.

Taken together, these data indicate that, in T-ALL, SCL may form aberrant protein complexes, therefore perturbing expression of genes essential for normal T-cell differentiation.

However, these aberrant SCL-containing complexes remain to be fully characterised and their exact molecular mechanism of action elucidated. Moreover, their relevance in human leukaemogenesis needs to be investigated.

4 SUMMARY OF THE INTRODUCTION

In summary, the haematopoietic system is an ideal model to begin to study some of the molecular mechanisms underlying the progressive restriction of the differentiation potential of stem cells and their progeny leading to production of lineage-specific mature cells.

Sequence specific transcription factors such as SCL play a pivotal role in this process. Their main function is to mark genes for either activation, through recruitment of co-activators like the chromatin modifying or remodelling complexes HAT and SWI/SNF, or repression, through recruitment of co-repressor complexes containing HDACs. They can also synergise with or directly antagonise the activity of other transcription factors.

Lineage specification requires the fine balance between activator and repressive functions of these regulators. Transcriptional programs essential for differentiation of a given lineage and leading to cell cycle arrest and production of structural and tissue-specific proteins, need to be activated in a timely fashion. Other programs, important for alternate lineage specification, have to be constitutively repressed. These different levels of regulation are likely to be achieved through the same set of tissue-specific transcription factors.

SCL has been described both as an activator and repressor of gene transcription in normal and malignant haemopoiesis. The molecular mechanisms conferring SCL function are largely unknown, but most likely involve interaction with protein partners. Therefore, the main part of my thesis work (Chapters IV, V and VI) deals with the identification and characterisation of SCL-containing multiprotein protein complexes to further elucidate the role of this transcription factor in lineage maturation.

5 STRUCTURE, HYPOTHESIS AND AIMS OF THIS THESIS

The results of this thesis are divided into four chapters (chapters III to VI). **Chapter III** describes work which was performed at the INSERM Unit 474 in Paris, France, under the direction of Paul-Henri Romeo. It describes the characterisation of a transgenic mouse model over-expressing SCL or its DNA-binding mutant under the control of a haematopoietic specific promoter, *sca-1*.

The aim of this first part of our work was to address three central questions surrounding SCL :

1. What is the role of SCL at the haematopoietic stem cell level ?
2. What exactly is its role in T-cell leukaemogenesis ?
3. Is the DNA binding activity essential for SCL function ?

When I arrived at INSERM Unit 474, work on this project had already started and transgenic mice had been generated. Possible perturbations in the stem cell pool were under investigation, but it soon became apparent that this transgenic model did not have any significant phenotype at the level of the adult haematopoietic stem cell. My project was to characterise transgene expression and abnormalities observed in B and T cell development. Unexpectedly, there was a severe defect in B lymphopoiesis. Besides, mice over-expressing SCL or its DNA-binding mutant did not develop T-cell leukaemia, but presented with a pre-leukaemic thymocyte phenotype. These results suggested that SCL over-expression under control of the *sca-1* promoter was not sufficient to cause leukaemia. This could have been due to inappropriate levels of SCL in the leukaemic target cell. Alternatively, SCL overexpression by itself may be insufficient and represent only the first step in the leukaemogenic process. Besides, DNA binding was dispensable for SCL's function in this setting, thus stressing the importance of protein-protein interactions for SCL function.

A logical progression ensuing from this work was therefore to try and identify protein partners of SCL.

Chapter IV to VI describe the work subsequently performed at the Molecular Haematology Unit, WIMM, Oxford. During this second and more significant part of my thesis, we aimed at identifying protein partners of SCL in definitive haematopoiesis.

As mentioned in the introduction, current data suggests that in definitive haematopoiesis the pentameric complex consisting of SCL/E2A, GATA-1, LMO-2 and LDB-1 can exert both repressor and activator functions dependent on cellular context and target genes involved. We therefore hypothesised that co-factors had to interact with this core complex to confer its function.

In an attempt to identify some of the proteins interacting with SCL, we established two different protein purification strategies using either conventional pre-purification followed by immunoprecipitation (**chapter IV**) or biotin/streptavidin-affinity purification whereby SCL was biotinylated *in vivo* and SCL-containing complexes pulled down by streptavidin-affinity (**chapter V and VI**). These protein complexes were subsequently identified by mass spectrometry. We have performed our analysis in both erythroid and megakaryoblastic cell lines as a first step towards the characterisation of protein complexes in two related but distinct haematopoietic cell types. This approach has allowed us to identify several new potential partners of SCL. So far, our work has focused on the validation and functional analysis of the interaction between SCL and one of these partners, the co-repressor ETO-2.

CHAPTER II : MATERIALS AND METHODS

1 MOUSE GENETICS PROCEDURES

This part of the work was performed in France. All procedures done on mice were authorized by the French authorities.

1.1 Mouse handling and breeding procedures

Mice were housed in a barrier facility with precautions to avoid introduction of pathogens (Hopital Henri Mondor, Creteil). Mice were bred in trios and offspring were weaned at 3-4 weeks after birth. The tip of the tails were removed for genotyping. Mice from 6-8 weeks of age were bred in groups of 2 or 3 females and 1 male. Timed mating was assessed by the presence of a vaginal plug, corresponding to 0.5 dpc (days post coitum).

1.2 Generation of DNA constructs and SCL and Δb SCL heterozygous mice

Ly-6E.1-hscl and *Ly-6E.1- Δb hscl* constructs were obtained by inserting the *human* (*h*) *scl* or *Δb hscl* c-DNA followed by the bovine growth hormone gene *polyA* into the poly IIIi vector (pL6C1a)²⁵⁷⁻²⁵⁹. The 14 kb *Ly-6E.1-hscl* and the 14kb *Ly-6E.1- Δb hscl* fragments were obtained by *BamH1* digestion. DNA fragments were purified by agarose gel electrophoresis and microinjected into the pronuclei of fertilised oocytes from a cross of B6D2F1 animals (C57BL/6 x DBA/2). Southern blot analysis of tail DNA was used to identify transgenic animals, to determine copy number and to assess integration patterns.

1.3 Genotyping of mice

Mice were genotyped by PCR. To obtain the genomic DNA, tails were lysed overnight at 55 °C in 500 μ l of lysis buffer containing 100 mM

Tris/200mMEDTA/0.5%SDS/proteinase K. DNA was extracted using Phenol-Chloroforme. Amplicates were electrophoresed on a 1.2% agarose gel.

2 CELL BIOLOGY PROCEDURES

2.1 Cell culture conditions and cell lines

Standard cell lines

MEL 585 (mouse erythroleukaemia cell line)⁴³, NIH 3T3 cells and the acute T cell leukaemia cell line Jurkat¹⁶⁷ were maintained in RPMI 1640 supplemented with 10% heat inactivated fetal calf serum (FCS), 2.0 mM L-glutamine, 100 units/ml penicillin, and 100 units/ml streptomycin.

MEL cell were induced to differentiate in 2% dimethyl sulphoxide (DMSO) for 3 to 5 days.

L8057 cells (murine megakaryoblastic cell line²⁶⁰) were grown in 50% IMDM, 50% RPMI 1640 with 15% FCS using the same supplements as described above.

All cells were cultured in a humidified atmosphere of 5% CO₂ at 37 °C.

ES cell *in vitro* differentiation assay

In vitro haematopoietic differentiation was performed as described^{89,261} and a general scheme of the rescue assay is described in figure 5.6. Briefly, SCL^{-/-} ES cells were expanded on STO feeder cells. SCL^{-/-} ES cell clones expressing biotinylated SCL were subjected to the first step of *in vitro* differentiation and permitted to form embryoid bodies (EB). To generate primitive erythroid colonies, day 6 EBs were disaggregated and replated into methylcellulose media containing erythropoietin (Epo, 2 U/ml). For definitive haematopoiesis, day 8 EBs were replated in methylcellulose in the presence of Epo (2 U/ml) and c-kit ligand (100

ng/ml) ; Interleukin (IL)-3 (1ng/ml), IL-6 (5 ng/ml), granulocyte colony stimulating factor (G-CSF, 30 ng/ml) and granulocyte-macrophage-CSF (GM-CSF 3 ng/ml); or c-kit ligand (100 ng/ml) and thrombopoietin (Tpo, 5 ng/ml) for definitive erythropoiesis, myelopoiesis and megakaryocytopoiesis, respectively. Colonies were scored on day 5 (primitive and definitive erythropoiesis) or day 7 (myelopoiesis and megakaryocytopoiesis). Colonies were then picked for analysis of cell morphology by May-Grunwald-Giemsa staining (according to the manufacturer's instructions) or RNA extraction.

2.2 Transfections

All transfections were performed using a BioRad Gene PulserElectroporator (BioRad, UK).

Stably transfected cells were selected in their respective medium containing puromycin (Sigma) 0.5 μ g/ml (Jurkat) or 2 μ g/ml (MEL and L8057) and zeocin (Invitrogen) 100 μ g/ml (MEL and L8057), 200 μ g/ml (SCL-/-) or 400 μ g/ml (Jurkat).

MEL

Stably transfected clones were obtained by electroporating 1×10^7 cells in 1 ml PBS 1X together with 15 mg of linearized cDNA. Electroporation conditions were : 220 V, 960 μ F. Cells were replated in RPMI 10%FCS for 48 hours before selection with the appropriate selection medium. Macroscopically visible clones were picked after one to two weeks and expanded.

L8057

5×10^6 L8057 cells were electroporated in 80 ml of PBS 1X with 15 mg of linearized cDNA using 0.2 mm electroporation cuvettes (EquiBio). Electroporation conditions were 250 μ F and 160 V. After 10 min of incubation on ice, cells were replated in 6

well plates for 48 hours before transfer into 50% methylcellulose (Methocult M3434, Stem Cell Technologies, Vancouver) containing the appropriate antibiotic.

ES cells

Undifferentiated ES cells were trypsinized and passed through a 19.5 Gauge syringe to obtain a single cell suspension. 1×10^7 cells were transfected with 20 μ g of linearized DNA in 1 ml of PBS. Electroporation conditions were : 800 V, 3 μ F. Cells were incubated at room temperature for 10 min and replated on feeder cells. After 48 hours, transfected cells were transferred onto gelatin coated dishes for selection. 9-10 days later, colonies of ES cells showing no sign of differentiation were picked and expanded.

2.3 Transactivation assays

NIH3T3 cells were transfected using the Lipofectamine 2000 reagent (Invitrogen) 24 hours after plating in 24-well plates at a density of 7×10^4 cells/well. Amounts of transfected DNA/well were as follows : 750 ng of luciferase reporter gene, 75 ng of SCL, E2A and GATA-1 and 375 ng of LMO2 and LDB-1 expression plasmids as previously described ¹⁸⁹, and 75 to 300 ng of ETO-2 expression vector. 50 ng of CMV-bGal was added to serve as transfection efficiency control. Total amounts of DNA were kept at 2 mg/well using pBluescript. 24 hours after transfection, cells were lysed and luciferase and bGal activities measured using standard procedures (kits from Roche). Each transfection was performed in duplicate and data presented are the results of 3 to 5 independent experiments.

2.4 Purification of mouse primary haematopoietic cells

Ter119+ splenocytes : C57Bl/6 mice were treated with phenylhydrazine (Sigma, 0.04 mg/g body weight). Three successive intraperitoneal injections were performed at 12 hour intervals. Spleens were harvested 6 days after the first

injection. Ter119⁺ cells were isolated after labelling the splenocytes with biotin-conjugated rat anti-mouse Ter119 antibodies (BD Pharmingen) and subsequent incubation with anti-biotin microbeads (Miltenyi Biotec). The positive fraction was recovered on AUTOMACS (Miltenyi Biotec). Purity was assessed by May-Grunwald-Giemsa staining.

Primary megakaryocytes

C57Bl/6 mice were treated with 5-fluorouracil (5-FU, 150 mg/kg, intraperitoneal). Eight days post-injection, mice were sacrificed, bone marrow cells harvested and replated at a density of 5×10^6 cells/ml in serum-free medium StemPro 34 (Invitrogen) in presence of Thrombopoietin (TPO, conditioned supernatant, 1/100 dilution). After 3 days of culture, megakaryocytes were isolated by negative selection. The culture was immunodepleted from Ter119-, Mac1-, Gr1- and B220-positive cells by labelling with the corresponding biotin-conjugated rat anti-mouse antibodies (BD Pharmingen), subsequent incubation with streptavidin microbeads (Miltenyi Biotec) and separation of the positive and negative fractions on MACS columns for large cells, according to manufacturer's instructions (Miltenyi Biotec). Purity of the negative fraction was assessed by May-Grunwald-Giemsa staining and flow cytometry (data not shown). The cell population was estimated to contain at least 95% of CD61⁺ megakaryocytes (data not shown).

Expansion and erythroid differentiation of wt fetal liver cells

After obtaining a single cell suspension, 1×10^6 cells/ml of fetal liver cells from day 12.5-13.5 mouse embryos were resuspended in serum-free stem cell expansion medium (StemPro-34TM Life Technology Gibco BRL) supplemented with human recombinant erythropoietin (2 U/ml), murine recombinant stem cell factor (100 ng/ml), dexamethasone (Sigma) (1 mM) and insulin-like growth factor 1 (IGF-1,

Biovalley, France) (40 ng/ml). The culture medium was changed daily for 3 days. On day 4, cells were resuspended in PBS 1X, EDTA, BSA at a concentration of $100 \text{ ml}/10^7$ cells and incubated with rat anti-Ter119 antibody (Becton Dickinson Pharmingen). After incubation on ice, cells were washed, incubated with anti-rat MACS beads (Miltenyi Biotec) and rotated at 4°C . Separation of the positive and negative fractions on MACS columns was achieved according to manufacturer's instructions (Miltenyi Biotec). Purity of the negative fraction was assessed by May-Grunwald-Giemsa staining and flow cytometry. Ter119 negative cells were used to prepare nuclear extracts. A fraction was induced to differentiate into erythroid cells using serum-free stem cell expansion medium (StemPro-34™ Life Technology Gibco BRL) supplemented with human recombinant erythropoietin (10 U/ml), insulin-like growth factor 1 (IGF-1, Biovalley, France) (40 ng/ml), human transferrin (Sigma) (1 mg/ml), Mifipristone (1 mM), FCS 10%, Biotin (Sigma) (0.1 mg/ml) and Hypoxanthine (Sigma) (5 mg/ml). Cells were harvested after 24 hours and 48 hours for preparation of nuclear extracts.

2.5 FACS analysis

Peripheral blood was taken from the tail vein and washed 3 times in PBS buffer. After incubation with the relevant antibodies, ready-made FACS lysis solution (Becton Dickinson) was added. Thymus, spleen and bone marrow were dissected out and homogenised in RPMI 1640 containing 1% fetal calf serum, $50 \mu\text{M}$ β -mercaptoethanol, 1% penicillin and streptomycin. Anucleated cells were lysed by osmotic shock in lysis buffer (NH_4Cl 155 mM, KHCO_3 10 mM, EDTA 0,1 mM) and washed twice with complete medium. Non-dissociated cells and tissue debris were filtered on a Cell strainer ($70 \mu\text{m}$ nylon) (Falcon, 35-2350). Cells were immunophenotyped using conjugated monoclonal antibodies and standard

techniques. The following antibodies were used: anti-CD4-FITC, CD8-PE, CD45R (B220)-FITC, CD45R (B220)-PE-Cy5, CD19-PE, IL-7R α -biotin, c-kit-APC, CD11b (MAC-1)-APC, CD11b-FITC, Sca-1-FITC and streptavidin-Cy-Chrome complex. All antibodies used were purchased from PharMingen. 10000 events were analysed on a FACScalibur (Becton Dickinson) permitting four-colour analysis.

2.6 Activation of B splenocytes

Splenic cells were depleted of T cells using anti-CD4 and anti-CD8 microbeads (Miltenyi Biotec) and activated for 3 days in α MEM containing 10% fetal calf serum, 50 mM β -mercaptoethanol, 1% penicillin and streptomycin and Lipopolysaccharide (25 μ g/ml).

2.7 Transplantation in NOD/SCID mice

Grafts were enriched for haematopoietic stem cells by treating transgenic and littermate control donors with 150 mg/kg of 5-Fluorouracil. On day 5 after treatment BM cells were harvested and depleted of CD4 or CD8 positive cells using α -CD4 and α -CD8 antibodies and magnetic beads (Miltenyi) to prevent graft versus host disease (GvHD). 5×10^4 cells were injected into the tail vein of NOD/SCID mice previously sublethally irradiated with 2.5 Grays. Weekly full blood counts were obtained starting at 6 weeks. BM cells were harvested from recipients 8 weeks after transplantation and haematological reconstitution was assessed by flow cytometry.

2.8 Cytology and Histology procedures

Thymus paraffin-embedded sections were prepared in the histopathology department of Henri Mondor Hospital. Sections were dewaxed and microwave

treated. HSCL protein was revealed using the mouse monoclonal antibody BTL-73 and the Mouse-on-Mouse Iso-IHC Kit (Inno Genex TH).

3 DNA PROCEDURES

3.1 Enzymatic modification of DNA

Restriction digestion of DNA was carried out using restrictions enzymes and corresponding buffers from New England Biolabs or Roche according to the manufacturer's instructions. In general, 5 units of enzyme were used to digest 2-10 μ g of DNA in a volume of 50-100 μ l for 1 hour to overnight.

When necessary, DNA ends of cloning vectors were phophatised with shrimp alkaline phosphatase (Roche).

DNA used for cloning or as probes for northern blotting was resolved on 1% agarose gels, and purified from the excised agarose slice using the QIAGEN Gel Extraction Kit according to the manufacturer's instructions.

3.2 Ligation and transformation of competent bacteriae

Vectors and inserts were ligated in a molar ration of 1:3 or 1:5 using the Rapid Ligation kit (Roche) according to the manufacturer's instructions. For transformation of purified plasmid vectors and freshly ligated DNA, 10^6 chemocompetent DH5 α bacteria were gently mixed with 200 pg of plasmid DNA or 10 μ l of the ligation reaction and incubated on ice for 30 minutes. The mixture was then exposed to 42 °C for 45 seconds and re-placed on ice for 2 minutes before adding 900 μ l of LB medium. The bacteria were incubated at 37 °C on a rocker for 30 minutes, pelleted and spread on LB agar plates supplemented with 50 μ g/ml of ampicillin. The cultures were then allowed to grow for 14 hours.

3.3 Constructs

The *Escherichia coli* BirA biotin ligase gene²⁶² was inserted as a PCR fragment (see primers xba-cla-birA 5' and xba-birA-R Table 2.1) into the human EF1 α promoter expression vector bearing a puromycin resistance gene. This antibiotic cassette was cloned into the vector using a polylinker (see Table 2.1). The SCL (wt and mutant) cDNAs were tagged in 5' with an oligonucleotide sequence encoding for the 23 amino acid biotinylation tag²⁶² using a PCR strategy. To do this, the first fragment, consisting of the biotag, was generated using oligo 2 and 3 (Table 2.1). The second fragment, SCL or mutant, was generated with oligo 1 and 4. The two fragments were then mixed, denatured and reamplified with oligos 3 and 4 and the PCR product cloned into the pEF1 α vector bearing a zeocin resistance gene. The mouse SSDP2²⁶³, mouse ETO-2²⁶⁴ and rat E2A²⁶⁵ (wt and mutants) cDNAs were subcloned into the pEF1 α -biotag vector in replacement of the SCL sequence using a PCR-based strategy. All PCR fragments were generated using the proof-reading Pfu-polymerase (Stratagene) and verified by sequencing.

3.4 Preparation of plasmid DNA

To obtain 5-50 μ g of plasmid DNA, a small-scale preparation of DNA was carried out using the QIAGEN Plasmid Mini Kit according to the manufacturer's instructions. To obtain 100-1000 μ g of plasmid DNA, a large-scale preparation of plasmid DNA was performed using the QIAGEN Plasmid Maxi Kit according to the manufacturer's instructions.

3.5 DNA sequencing

Automated DNA sequencing using the 3100 Genetic Analyser (Applied Biosystems) was carried out by the MHU Sequencing Facility (MHU, WIMM).

Table 2.1 **name of oligo** **sequence**

| | | |
|---------------|-------------------------|---|
| biotag system | Biotagged-SCL | |
| | oligo1 | 5' GAG GAG GAC CTG GGC ACG GAG CGG CCG CGG AGC GAG 3' |
| | oligo2 | CGG CGG CCG CTC CGT GCC CAG GTC CTC CTC CGA GAT |
| | oligo3 | GCT CTA GAC TCG AGA CTC GTC ATA CCA CTA |
| | oligo4 | GCT CTA GAG TTA ACT CAC CGG GGG CCA GCC CCA TC |
| | semi quant reverse | CCG GAA TTC CGC GCC GCA CTA CTTT GGT GTG |
| | semi quant forward | CGC GGA TCC ACG GAG CGG CCG CGG AGC GCG |
| | Bir A | |
| | xba-cla-birA 5' | GCT CTA GAC CAT CGA TAT GAA GGA GGA TAA CAC CGT GCC ACT G |
| | xba-birA-R | GCT CTA GAG CTT TAT TTT TCT GCA CTA CGCAGG GAT ATT TC |
| | Bir 650 | CGG TAT CGT GAT GGC GGA AGT |
| | Biotagged-SSDP-2 | |
| | Forward Not-SSDP | ATA AGA AAT GCG GCC GCC GTA CGG CAA AGG CAA G |
| | Reverse Xba-SSDP | GCTVCTA GAT CAC ACG CTC ATT GTC ATG CTA GG |
| | Polylinker | |
| | Cla-1-Sal-1-Asp718F | CGA TAC GGT CGA CTG GG |
| | Cla-1-Sal-1-Asp718R | GTA CCC CAG TCG ACC GTA T. |
| real time PCR | | |
| | hprt 5' | GCT GGT GAA AAG GAC CTC T |
| | hprt 3' | CAC AGG ACT AGA ACA CCT GC |
| | hscl 5' | GCC GGA TGC CTT CCC TAT GTT |
| | hscl 3' | AAT GAC CAG GAG GAG GAG GG |
| | mtrc 5' | ACA GAC AGC ATT CTG GGA AAG C |
| | mtrc 3' | ATC TTG CAG GTG AAG CTT GT |
| | pTa 5' | TCA GGT GTC AGG CTC TAC GA |
| | pTa 3' | CCC ATA GGT GAA GGC GTC TA. |

3.6 Polymerase chain reaction

PCR reactions were carried out in order to genotype mice, to generate constructs and to obtain specific DNA probes for Northern blotting. For primers : see table 1. In general, PCR reactions were carried out in a total volume of 50 μ l, containing 5 ng of template, 5 μ l of DMSO, 5 μ l of 10X enzyme buffer, 10 mmol of each nucleotide, 25 pmol of each oligonucleotide and 1 unit of AmpliTaq Gold DNA polymerase (Applied Biosystem) or Pfu (Stratagene). Conditions for PCR of SCL cDNA were as follows : 94 °C for 3 minutes, 95 °C for 1minute, 58 °C for 1 minute, 72 °C for 1 min, 20 cycles, 72 °C for 10 min.

4 RNA PROCEDURES

4.1 Preparation of total RNA

Total RNA of spleen, thymus, kidney, liver were prepared with TRIZOL (Gibco, BRL). Total RNA of cell lines or ES cell derived haematopoietic colonies was obtained using the QIAGEN Rneasy Kit according to the manufacturer's instructions.

4.2 Northern Blotting

For Northern Blots, 10-15 μ g of total RNA in RNA loading buffer were separated on a 1% formaldehyde agarose gel containing ethidiumbromide in 1X MOPS running buffer at constant 100 V and transferred to a Nitrocellulose Hybond N+ membrane (Amersham Biosciences) by capillary transfer using 20X SSC buffer (0.3 M sodium citrate, 3 M NaCl; pH 7.0) overnight. The membrane was cross-linked using a Stratalinker UV Crosslinker (Stratagene).

The membrane was pre-hybridised for 30 minutes in ExpressHyb(Clontech) containing denatured salmon DNA (Invitrogen). Hybridisation was performed using the same buffer containing the denatured radioactive probe for 1 hour at 65 °C in a rotary oven. Membranes were then washed three times at room temperature with 2X SSC containing 0.1% SDS for 15 minutes, followed by 2 washes at 50 °C for 20 minutes in 0.1X SSC with 0.1% SDS. Probes used for northern blotting were excised from cDNA plasmids (SCL) or were generated by PCR using cDNA plasmids as a template and sequence specific PCR primers (Bir A) (Table 2.1). Radioisotope labelled DNA probes were prepared by random hexamer method using the Megaprime DNA labeling kit according to the manufacturer's instructions (Amersham Biosciences).

Membranes were exposed to autoradiographic films at –80 °C overnight.

4.3 Semi-quantitative RT-PCR

SCL expression was quantitated by RT-PCR on RNA isolated from haematopoietic colonies derived from wt ES cells and SCL^{-/-} ES cells transfected with Bir A and biotagged SCL and cDNA prepared according to standard protocols. One-tenth of the synthesized cDNA was used for PCR (same protocol as described above). Each PCR reaction contained 0.1μCi α-32P dCTP. Two sets of primers were added : one designed to amplify 570bp of the SCL sequence (see table 2.1) and one designed to amplify 249bp of the constitutively expressed hypoxanthine phosphoribosyl transferase (HPRT) transcripts⁹⁰. Aliquots were analysed on a 4% PAGE after cycles 18-24 for HPRT and SCL sequences. Quantification was carried out using the Phosphorimager (Storm, Molecular Dynamics). Control experiments without reverse transcriptase in the cDNA synthesis reactions did not show specific PCR products.

4.4 Quantitative real-time PCR

For quantification of transgene expression in different haematopoietic lineages and animal lines, bone marrow cells and thymocytes were sorted by flow cytometry using anti-B220-PE-Cy5 (CD45R), anti-TER-PE, anti-CD11b-FITC (MAC), anti-CD4-FITC and anti-CD8-PE antibodies (all from PharMingen). After TRIZOL extraction of total mRNAs and standard reverse transcription, quantitative PCR was performed with the LC FastStart DNA Master SYBR Green I reaction kit (Roche Diagnostics, cat no: 2239264). The primers used are indicated in Table 2.1. MgCl_2 was used at a final concentration of 4 μM . The annealing temperature was 60°C. All samples were run in duplicates. Melting Curve analysis and quantification calculation was performed using fluorimetric online detection with the LightCycler (Roche Diagnostics).

5 PROTEIN PROCEDURES

5.1 Preparation of nuclear extracts

Small scale preparation of nuclear extracts

10^7 cells were washed in 1 ml of PBS 1X and pelleted into an eppendorf tube. After resuspension in 10 times the pellet volume of buffer A (10 mM Hepes pH 7.9, 1.5 mM MgCl_2 , 10 mM KCl, 0.5 mM PMSF or a protease inhibitor cocktail (Complete EDTA-free, Roche) and 0.5 mM DTT) cells were kept on ice for 10 min and vortexed. Nuclei were pelleted for 10 sec at maximum speed and resuspended in 5 times the pellet volume of buffer C (20 mM HEPES pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl_2 , 0.2 mM EDTA, 0.5 mM PMSF or protease inhibitor

cocktail (Complete EDTA-free, Roche) and 0.5 mM DTT). After incubation on ice for 20 min and centrifugation, supernatants were kept for protein analysis.

Large scale preparation of nuclear extract

40 ml of confluent cells were aliquoted into 20 flasks containing 100 ml of medium each. 48 hours later, a further 150 ml of medium was added. 72 hours later, a 5 ml cell pellet corresponding to approximately 2×10^9 cells was harvested by spinning for 15 min at 3000 rpm and washed once in PBS1X. After measuring the packed cell volume pellets were resuspended in 10 pellet volumes of buffer A (see above). Cells were spun down immediately at the same conditions, resuspended in buffer A and transferred into the loose Dounce homogenizer. After spinning the homogenate for 1 hour at 20000 RPM and 4 °C in the ultracentrifuge using the SW28 rotor, the nuclear pellet was resuspended in 8 ml of 150 mM Heng buffer (150 mM KCl, 20 mM Hepes, 20% Glycerol, 0.25 mM EDTA, 0.05% NP40). 1 ml of 2.2 M KCl Heng buffer was added dropwise to achieve a final salt concentration of 420 mM KCl and nuclei were homogenized using the tight Dounce homogenizer. The homogenate was spun at 40000 RPM for 1 hour at 4 °C, the supernatant aliquoted and protein concentration dosed with Bradford reagent (Biorad).

5.2 Sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

For home made gels, 30% (w/v) acrylamide : 0.8 (w/v) bis-acrylamide stock solution (BioRad) was mixed with the separating (375 mM Tris-Cl pH 8.8, 0.1% SDS) and stacking (125 mM Tris-Cl pH 6.8, 0.1% SDS) gel buffers to obtain a final concentration of 10% versus 3%, respectively. Gels were polymerized by the addition of 0.05% ammonium persulphate (APS) and 1% of N,N,N',N'-tetramethylethylenediamine (TEMED) for 30 minutes at room temperature.

Electrophoresis was performed in Tris-glycine running buffer (25 mM Tris-base, pH 8.3, 190 mM glycine, 1 mM EDTA, 0.1% SDS) at constant 80 V. Gel electrophoresis was performed using the Mini gel electrophoresis system (Hoeffer) under denaturing conditions. Protein samples were mixed in 2X Laemmli sample buffer and 0.5 mM DTT and boiled at 95 °C for 5 minutes just before loading.

Precast gel electrophoresis using 4-12% Bis-Tris and 7% Tris-Acetate gels was performed in the NuPage electrophoresis system (Invitrogen) according to the manufacturer's instructions. The molecular weight in kD was estimated using the See Blue Marker from InvitroGen.

5.3 Western Blotting

Proteins resolved by SDS-PAGE were electrotransferred onto Hybond-P membranes (Amersham Biosciences) using a wet blotting method. Transfer was performed in 25 mM Tris, 192 mM Glycine, 20% Methanol at constant 25 V overnight at 4 °C. Subsequently, membranes were incubated for 1 hour at room temperature in TBST (50 mM Tris, 150 mM NaCl, 0.1% Tween 20) containing 5% semi-skimmed milk in order to block non-specific protein binding sites. Next, membranes was incubated for 2 hours at room temperature with primary antibodies diluted in TBST containing 5% semi-skimmed milk. Primary antibodies and dilutions are indicated in Table 2.2.

After incubation with primary antibodies, membranes were washed 3 x 20 minutes in TBST before incubation with HRP-conjugated secondary antibodies diluted in TBST (Table 2.2). After washes, immunoblots were developed using enhanced chemiluminescence (ECL, Amersham, UK). If indicated, filters were stripped in 0.5M Tris pH 6.8, 20% SDS, 14.3 M β -mercaptoethanol for 30 minutes at 50 °C

Table 2.2

| | cat no | origin | antigen | source | concentration | dilutions | IP conditions |
|---------------------|----------------|-----------------|-----------------|-------------------|---------------|-----------|-------------------|
| primary Ab | sc 318 (C-18) | Santa Cruz | p107 | rabbit polyclonal | 200ug/ml | WB 1:200 | IP: 10ul |
| | sc-357 (A-20) | Santa Cruz | HEB | rabbit polyclonal | 200ug/ml | WB 1:200 | |
| | sc-762 (H-208) | Santa Cruz | E12 | rabbit polyclonal | 200ug/ml | WB 1:200 | |
| | sc-349 (V-18) | Santa Cruz | E2A, E12/E47 | rabbit polyclonal | 200ug/ml | WB 1:200 | IP: 10ul |
| | sc-763 (N-649) | Santa Cruz | E47 | rabbit polyclonal | 200ug/ml | WB 1:200 | |
| | | gift: S Orkin | Ldb-1 | rabbit polyclonal | | WB 1:1000 | |
| | | gift S Orkin | Lmo-2 | rabbit polyclonal | | WB 1:1000 | |
| | | gift: S Orkin | Gfi-1b | rabbit polyclonal | | WB 1:1000 | IP: 10ul |
| | sc-994 (K-20) | Santa Cruz | mSin3A | rabbit polyclonal | 200ug/ml | WB 1:5000 | |
| | sc-11417(H-99) | Santa Cruz | HDAC 3 | rabbit polyclonal | 200ug/ml | WB 1:200 | |
| | sc-9741(G20) | Santa Cruz | ETO-2 | goat polyclonal | 200ug/ml | WB 1:200 | IP: 20ul |
| | sc-265 (N6) | Santa Cruz | GATA-I | rat monoclonal | 200ug/ml | WB 1:500 | IP: 50ul + bridge |
| | sc-1234 (M20) | Santa Cruz | GATA-I | goat polyclonal | 200ug/ml | WB 1:200 | |
| | sc-1609 | Santa Cruz | NCo-R | rabbit polyclonal | 200ug/ml | WB 1:200 | |
| | sc-585 | Santa Cruz | p300 | rabbit polyclonal | 200ug/ml | WB 1:200 | |
| | home made | | murine SCL CT | rabbit polyclonal | | WB 1:1000 | IP: 20ul |
| | BTL 136 | gift: K Pulford | murine/humanSCL | monoclonal | | WB 1:100 | |
| | BTL 73 | gift: K Pulford | human SCL | monoclonal | | WB 1:100 | |
| normal IgG | sc-2026 | Santa Cruz | rat IgG | rat | 0.5ug/ul | N/A | 4ul |
| | sc-2027 | Santa Cruz | rabbit IgG | rabbit | 0.5ug/ul | N/A | 4ul |
| | sc-2028 | Santa Cruz | goat IgG | goat | 0.5ug/ul | N/A | 4ul |
| secondary Ab | | Jackson | Protein G-HRP | N/A | N/A | WB 1:3000 | |
| | | Jackson | Protein A HRP | N/A | N/A | WB 1:3000 | |

and washed twice at room temperature for 10 min in TBS, 0.1% Tween-20. Filters were stored at 4 °C.

5.4 Quantitative Western Blots

Nuclear extracts were prepared as described. Total protein in nuclear extracts from transfected clones and wild type cells was dosed after the Bradford method and loaded onto a 10% SDS-Page gel. After transfer, the membrane was cut into two horizontal parts. The upper part containing molecular sizes above 30 kD was incubated with anti-SCL polyclonal rabbit serum, the lower part with anti-Grb-2 as a loading control. After washing in TBST and incubation with a HRP-conjugated secondary antibody, signals were revealed by chemoluminescence (Amersham). Signal intensities were quantitated using the Quantity One software (Biorad).

5.5 Gel filtration

Crude nuclear extracts (25 mg total protein) were precipitated with 55% saturated ammonium sulphate, and the precipitated proteins redissolved in 20 mM Tris pH 8, 200 mM NaCl, Glycerol 10%, 0.05% Triton. The concentrated nuclear extracts were fractionated on a Superose 6H/R column (Amersham Biosciences) in the same buffer. Fractions of 0.5 ml were collected at a flow rate of 0.4 ml/min. Individual fractions were then subjected to western blotting.

5.6 Affinitypurification of anti-SCL antibodies

Polyclonal rabbit SCL antibodies were raised against the C-terminal and the N-terminal regions of the protein^{186,188}. For affinitypurification of these antibodies, cDNAs coding for these regions were subcloned into the pGEX2TK GST expression vector using a PCR-based strategy. The constructs were transformed into DH1α and expression of the fusion proteins was achieved by adding 0.2 mM IPTG to 500 ml of bacterial culture. After 4 hours, bacteriae were pelleted and

lysed in lysis buffer containing 50 mM Tris HCl pH 8, 1 mM EDTA, pH 8%, 50 mM NaCl and 10 mg/ml lysozyme. Lysates were sonicated, spun at 10000 RPM for 10 min and the supernatants were incubated with Gluthathione-Sepharose 4b slurry to allow adherence of the peptide to the beads.

Covalent binding

Sepharose beads were resuspended in 0.2 M Sodium Borate pH 9 and covalent binding of the GST fusion proteins was achieved upon incubation for 30 min in 20 mM dimethylpimelimidate (DMP). Reactions were quenched with 0.2 M ethanolamine for 2 hours. Beads crosslinked to the Ct and Nt peptides were loaded separately onto two columns (BioRad 64775, cat no : 731-1550, 0.8x4 cm Polyprep chromatography column) and stored in 0.02% Sodium Acide/PBS.

Prior to affinitypurification, antisera were precleared from anti-GST antibodies using immobilized GST columns (Pierce, cat no : 20505). Briefly, after equilibration of the column with PBS1X, 4 ml of each antiserum were mixed with 4 ml of PBS1X and loaded onto the column. An additional 10 ml of PBS1X were applied and fractions collected and pooled according to their UV absorbance at 280nm.

For affinitypurification, columns containing the Ct and Nt antigens were washed in 10 mM Tris pH 7.5, 100 mM Glycine pH 2.5, 10 mM Tris pH 8.8, 100 mM Triethylamine pH 11.5 and 10 mM Tris pH 7.5 until the pH reached 7.5. Antisera depleted from anti-GST antibodies were applied to the column, which were then washed with 10 mM Tris pH 7.5 and 10 mM Tris/500 mM NaCl. Antibodies were eluted using 100 mM Glycine pH 2.5 and neutralized with 1 M Tris pH 8 containing 1 mg/ml BSA. The samples were concentrated on a CENTRIPLUS YM 10 spin column (Centricon).

5.7 Immunopurification of SCL-containing complexes

25 μ l of protein G beads (P3296 Fast Flow, Sigma) were washed twice in 100 mM Sodium Borate pH 9 prior to saturating them with 20 μ l of Ct or Nt immunopurified antibodies for 1 hour at room temperature. The antibodies were crosslinked to beads as described above using DMP and ethanolamine. Crosslinked beads were washed twice in Tris/Saline pH 8, 0.05% Triton, once in 100 mM Glycine pH 2.5 to remove unbound antibodies and three more times in Tris/Saline pH 8, 0.05% Triton.

Subsequently, beads were incubated overnight with 100 μ l of gelfiltration fraction on a rotating wheel at 4 °C.

Beads were washed four times in Tris/Saline pH 8, 0.05% Triton, 0.5 mM PMSF, 0.5 mM DTT and elution of SCL containing complexes was carried out with 100 mM Glycine pH 2.5 neutralized by 1 M Tris pH 9.

Eluates were diluted in Laemmli buffer and 0.5 mM DTT and loaded on a 4-12% SDS-Page gel for Western Blot and Silverstain.

5.8 Affinitypurification of SCL-containing complexes

Maxi pull downs were achieved by incubating 5 mg of crude nuclear extract with 100 μ l of paramagnetic streptavidin beads (Dynabeads M-280, Dynal (Great Neck, NJ). Beads were washed prior to use with TBS1X and 200 μ g/ml of ovalbumin (Sigma). Nuclear extracts were diluted in binding buffer containing TBS1X, 0.3% Nonidet P-40 to obtain a final salt binding concentration of 150 mM NaCl. After incubation overnight on a rotating wheel at 4 °C beads were washed 6 times in washing buffer (TBS1X, 0.3% Nonidet P-40, 250 mM NaCl) and then boiled to

elute the complex. Samples were resolved on a 4-12% SDS Page gradient gels for Coomassie stain and Western Blots.

5.9 Protein staining procedures

Silverstaining was performed using a kit (Invitrogen) according to the manufacturer's instructions. Bands were destained immediately after cutting.

Sypro Ruby Protein gel staining (BioRad) and Colloidal Coomassie staining (Invitrogen) were used according to the manufacturer's instructions.

5.10 Mass Spectrometry

Proteins eluted from the beads were separated on a 4-12% SDS PAGE gel and stained with Colloidal blue (Invitrogen). The entire lane was cut into at least 25 slices and subjected to trypsin digestion using either a MWG Roboseq 4204 or a Qiagen 3000 robot. Analysis of tryptic digests was performed on a MicroMass Q-TOF Global with a capillary HPLC system with a nanospray probe. 5 µl of sample were run on a long column to separate out the peptides. Database searches were performed with MASCOT using the following settings: fixed modifications: Carbamidomethyl (C), variable modifications: Oxidation (M), peptide charge 2+ and 3+. The data format was pkl www.matrixscience.com.

5.11 Co-immunoprecipitation and Depletion Experiments

Nuclear extracts were diluted in TBS 1X/0.3% Nonidet P-40 to obtain a final concentration of 150 mM NaCl and precleared for 30 min at 4 °C with 2 µg of normal IgG (Santa Cruz: rat sc-2026, rabbit sc-2027, goat sc-2028) together with 25 µl of Protein G beads (Fastflow, Sigma). Beads were pelleted and kept as a control. The supernatant was incubated overnight at 4 °C with primary antibody and 25 µl of Protein G beads. For immunoprecipitation with anti-GATA-1 N6

antibody, a rabbit anti-rat bridging antibody (Jackson Immunodiagnostics) bound to beads was used. After completion of immunoprecipitation, IgG control and sample beads were washed 4 times in TBS 1X, 0.3% Nonidet P-40, 250 mM NaCl and boiled in 2x Laemmli buffer. For depletion experiments, the supernatant of the first immunoprecipitation was subjected to a second round of immunoprecipitation. Protease inhibitors (Complete EDTA-free (Roche)) were present throughout these procedures.

5.12 Immunolabelling and colocalisation

MEL and L8057 cells on coverslips were fixed in 4% paraformaldehyde and antigens indirectly immunolabelled with monoclonal mouse antibodies directed against SCL (1/100 dilution) and SC35 (1/100 dilution), rabbit polyclonal Ldb1 antibodies (1/100 dilution), or goat anti-ETO-2 antibodies (1/20 dilution). Secondary antibodies used were Alexa Fluor 488-conjugated donkey anti-mouse (Molecular Probes), Alexa Fluor 546-conjugated donkey anti-goat (Molecular Probes) and Cy-3-conjugated donkey anti-rabbit (Jackson Laboratory). Nucleic acids were counterstained with 1 µg/ml DAPI (data not shown), monochrome images collected using a Radiance 2000 MP confocal microscope (BioRad), intensities over the nucleoplasm and equivalent areas of the slide measured using the metamorph software and data exported to Excel for background subtraction and analysis²⁶⁶. Images were exported to Adobe Photoshop, contrast stretched and pseudocolored images were generated.

CHAPTER III: CHARACTERISATION OF A TRANSGENIC MOUSE MODEL OVER-EXPRESSING SCL OR ITS DNA BINDING MUTANT UNDER THE CONTROL OF THE SCA-1 PROMOTER

1 INTRODUCTION

1.1 The *sca-1* promoter

The *Ly-6E.1* and *Ly-6A.2* genes are highly homologous strain-specific alleles of the mouse belonging to the *Ly-6* multigene family and encode 876 and 830 base transcripts, respectively, and a 10-12 kD GPI-linked cell surface glycoprotein called Sca-1²⁶⁷. The proteins are identical in sequence except for two aminoacids. Little is known about *Ly-6E/A* expression during mouse embryonic development. Yolk sac cells from embryos at 10-11 dpc have been shown to be negative for *sca-1* expression as measured by RT-PCR and flow cytometry, however, the intraembryonic AGM region expresses *Ly-6E/A*². During adult haematopoiesis, the *Ly-6E/A* gene is expressed in the haematopoietic stem cells (HSCs) of mouse bone marrow and fetal liver as well as in multipotent progenitors and mature T and B lymphocytes^{258,259}. However, the expression pattern is complex and allele-specific in inbred strains of mice. For example, *Ly-6A.2* strains express Sca-1 on 99% of stem cells with repopulating activity, whereas *Ly-6E.1* strains express Sca-1 on only 25% of these cells²⁵⁷. Transgenic mice studies, using a *Ly-6E.1-LacZ* construct, have shown that a 14 Kb *Ly-6E.1* genomic fragment is sufficient to recapitulate endogenous *sca-1* gene expression²⁵⁷. We therefore used these *sca-1* gene regulatory sequences to study the effects of forced hSCL expression on

lymphocyte development and to understand how ectopic expression of hSCL or its DNA-binding mutant causes T cell leukaemia.

1.2 T cell lymphopoiesis

The characterisation of T cell developmental stages is based on the rearrangement status of the TCR loci and the expression of the CD4 and CD8 co-receptors (see figure 3.1). The earliest T cell progenitors in the thymus are present within the CD4 and CD8 double negative (DN) population. In murine models, this DN population can be further subdivided into four developmental stages DN1 to 4 according to CD44 and CD25 surface marker expression. CD44⁺ and CD25⁻ still have the ability to mature into NK and T lineage cells. Commitment to the T lineage is largely associated with activation of CD25 expression and downregulation of CD44 and is followed by initiation of gene rearrangements at the TCR β , γ and δ loci. Formation of a functional TCR β gene product allows expression of the pre-TCR complex, which includes the TCR β chain as well as the pre-T α protein and the CD3 assembly of signaling molecules. Signaling mediated by the pre-TCR complex results in developmental progression, also referred to as β selection. This transition is characterised by inhibition of gene rearrangement, initiation of cellular expansion, and maturation into CD4 and CD8 double positive (DP) thymocytes. DP cells then exit the cell cycle and begin TCR α gene rearrangement. The expression of an $\alpha\beta$ TCR allows DP cells to undergo major histocompatibility complex (MHC) mediated positive or negative selection. Positively selected DP thymocytes downregulate either CD4 or CD8 expression to become single positive (SP) mature T lineage cells ²⁶⁸.

Important transcription factors involved in the differentiation of T cells are the bHLH proteins encoded by the E2A gene, E12 and E47. Whereas DN1 thymocytes express little or no E2A, it is upregulated during the transition of the DN2 to DN4 stage. However, upon pre-TCR mediated signaling, E47 DNA-binding activity is significantly downregulated. As compared to DN thymocytes, DP cells express lower levels of E47 proteins, which are decreased further during the transition from DP to SP cells. These observations indicate, that a gradient of E47 expression is present during thymocyte development and that downregulation of this protein is required to promote the maturation of DN cells to the DP stage and further to the SP stage²⁶⁹.

1.3 B lymphopoiesis

As T cells, B cells develop from the CLP. The different stages of B cell differentiation can be subdivided according to surface marker expression and rearrangement of Immunoglobulin chains (figure 3.1). CLP and early pro-B cells express interleukin 7 receptor (Il-7R) α and c-kit on their surface. Subsequent expression of the B cell marker B220 initiates pro-B cell differentiation marked by CD19 expression and completion of D_H-J_H rearrangement. Productive V_H-DJ_H recombination in late pro-B cells results in cell surface expression of the Ig μ proteins as part of the pre-B cell receptor (pre-BCR). Signaling through the pre-BCR promotes light-chain gene recombination and subsequently leads to the emergence of immature IgM positive B cells that emigrate from the bone marrow to the peripheral lymphoid organs (reviewed in²⁷⁰). Precursor commitment and progression through all stages of B lineage development as delineated above is dependent on specific gene expression programs as well as environmental cues.

The analysis of genetically altered mice has led to the identification of a number of transcription factors, including E2A, EBF and Pax 5, that regulate many B lineage specific genes. These transcription factors act in a cascade and in synergy to establish and maintain the expression of target genes that promote the B lymphocyte maturation program from the common lymphoid progenitor up to the isotype switching of activated B lymphocytes. In the following I would like to focus on the role of E proteins in B cell development.

Considering that E2A proteins are components of heterodimers that are important for the development of many lineages, it was surprising that the targeted deletion of the E2A gene did not lead to any defects in these lineages, suggesting that the alternative class I bHLH proteins HEB and E2.2 are able to compensate for the loss of E2A^{100,101}. However, in B cell development where E2A proteins form homodimers, a severe defect was observed in E2A deficient mice^{100,110}.

E2A-deficient mice display a complete block in B lineage development prior to the onset of IgH DJ rearrangement^{100,101}. Early B lineage specific transcripts, including Igμ, Igκ, RAG1 and Pax5, are lacking in E2A-null mutant bone marrow cells. Two other E protein members, E2-2 and HEB, also play a role in B lineage development. Mice deficient for these proteins generate mature B cells but contain 50% fewer pro-B cells in the fetal liver, and mice that are transheterozygous for any two of the four E proteins display fewer pro-B cells than mice heterozygous for any E protein alone²⁷¹. Restoration of E protein expression in an E2A null background allows for B cell development albeit at varying degrees. Expression of both E12 and E47 transgenes in E2A-deficient mice promotes B lineage development better than either transgene alone¹¹⁰. Furthermore, expression of two, but not one, copies of the HEB gene introduced into the E2A locus results in a

partial rescue of B cell development²⁷². Mature B cells are generated, but wild-type numbers are not achieved. Therefore, the overall timing and dosage of E protein activity may be a key determinant in B lineage progression rather than the activity of a specific E protein. More recent work using an affinity-tagged E2A knock-in allele and ChIP experiments has identified a number of critical early B-lineage genes as direct targets of E2A^{273,274}.

In addition to this pivotal role in B cell commitment and early differentiation, E proteins have also been implicated in the initiation of V(D)J recombination and IL-7 dependent expansion and survival of pro-B lymphocytes²⁷⁵ as well as pro-B to pre-B cell transition. Besides, E2A protein expression is high in activated mature B cells and cells present in the dark zone of germinal centres^{276,277}, where they undergo clonal expansion, affinity maturation and immunoglobulin isotype switching. Although loss of E2A activity in the germinal centre does not interfere with proliferation and survival, it abolishes completely isotype switch recombination²⁷⁶.

2 OUTLINE OF EXPERIMENTS

The aim of the experiments presented in this chapter was to further establish the role of ectopic SCL and its DNA binding mutant Δb SCL in lymphopoiesis and leukaemogenesis. Using a transgenic mouse model expressing SCL under the control of the *Ly-6E 1* promoter we hoped to address the hypothesis that over-expression of wt or Δb SCL in the stem cell compartment and in early thymocytes would perturb the stem cell pool and generate a pre-leukaemic or leukaemic phenotype.

We established a transgene expression profile using RNA and protein prepared from various haematopoietic and non-haematopoietic organs. Besides, we immunologically labelled histological sections of the thymus to assess ectopic SCL expression. To quantitate transgene levels, we undertook real-time PCR analysis of bone marrow cell.

Next, we described the haematopoietic phenotype by performing blood films, full blood counts and FACS analysis of peripheral blood, bone marrow and thymus. Moreover, we obtained histology of peripheral lymphoid organs.

In order to characterise the defect observed in T- and B-cell differentiation more closely, we sorted progenitor populations from thymus and bone marrow and performed RT-PCR of genes critical for normal T- and B-cell differentiation.

Furthermore, we extended our analysis to mature B-cells and induced isotype switching *in vitro*. Besides, we performed transplantation experiments in NOD/SCID mice with subsequent FACS analysis of peripheral blood and bone marrow to confirm that the defects observed were cell-intrinsic.

The results of this analysis indicate that over-expression of SCL or Δb SCL driven by the *Ly-6E.1* promoter does not lead to T-cell leukaemia, but induces a profound defect in T lymphopoiesis. Surprisingly, we also found a block in B lymphopoiesis leading to a severe reduction in immature and mature B cells and a defect in isotype switch recombination. Mice expressing the SCL DNA-binding mutant developed the same phenotype as wild-type SCL in T- and B-cells, implying that the underlying mechanism of action in this setting was DNA-binding independent.

Taken together, these data indicate that SCL or Δb SCL under the control of the *Ly-6E 1* promoter leads to an arrest of normal lymphoid differentiation, suggesting that

they may sequester or repress important lymphoid transcription factors such as the E proteins.

3 RESULTS

In the ensuing result section, I will present the T-and B cell phenotype.

3.1 Phenotype of *Ly-6E.1-hscl* and *Ly-6E.1-Δbhscl* transgenic mice

Transgenic constructs were made by inserting the *human SCL* (*hscl*) cDNA or the *human SCL* cDNA lacking the DNA binding domain (*Δbhscl*) followed by the bovine growth hormone gene polyA sequence into the *Cla* I site of the 14 Kb *Ly-6E.1* cassette (fig. 3.2A)^{258,259}. The *Δbhscl* mutant was kindly given to us by Dr E MacIntyre (Hopital Necker-Paris) and was derived using oligonucleotide-mediated mutagenesis to delete the basic domain containing the amino acids 187 to 199 as previously described^{210,278}. The *Ly-6E.1-hscl* or the *Ly-6E.1-Δbhscl* fragments were microinjected into the pronuclei of fertilised (C57BL/6 x DBA/2) F1 oocytes. Two transgenic lines were generated with the *Ly-6E.1-hscl* construct, line 6 (L6) and line 8 (L8). Southern blot analysis of tail DNA showed that L6 contained 10 copies of the transgene while L8 contained 2 copies (data not shown). While the L6 females were infertile, making it impossible to generate a homozygous line 6, we could obtain, at low frequency, homozygous descendants for line 8. Generation of *Δbhscl* transgenic mice was difficult because of low transgene transmission. One founder did not transmit while another passed on the transgene to only one out of 40 offspring. The third founder transmitted the transgene to 10 out of 68 descendants establishing the transgenic line Δb L3 containing 6 copies of the transgene (data not shown). As we could only generate one *Δbhscl* transgenic

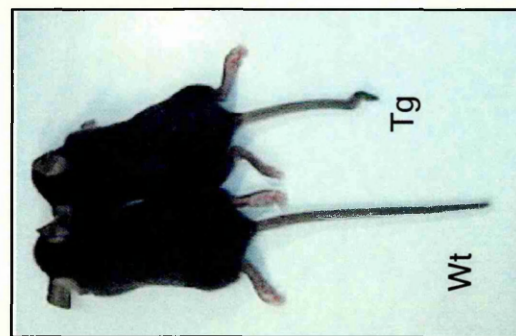
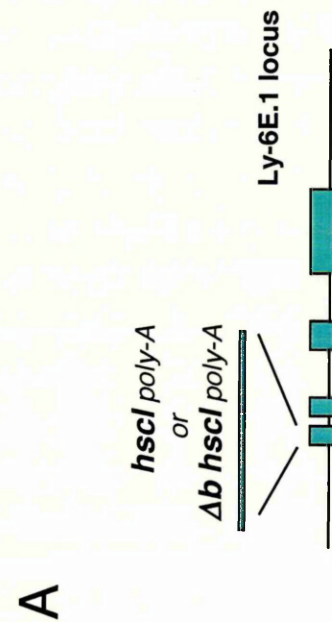


Figure 3.2: *Ly-6E.1 hsc1* and *Ly-6E.1 Δbhsc1* vectors and transgene expression

A. The *Ly-6E.1* vector contains the 14Kb *Ly-6E.1* genomic sequence including promoter and 3' sequence of the *Ly-6E.1* gene. A human (*h*) *scl* cDNA or *Δbhsc1* cDNA were linked to the bovine growth hormone polyA sequence and inserted in the first exon of the *Ly-6E.1* gene as previously described . The *ΔbSCL* protein lacks the basic domain containing aminoacids 187 to 199 . **B.** Photo of wild type (left) and a representative high copy transgenic adult mouse (right) showing the shorter length and kinked tail of the transgenic mice.

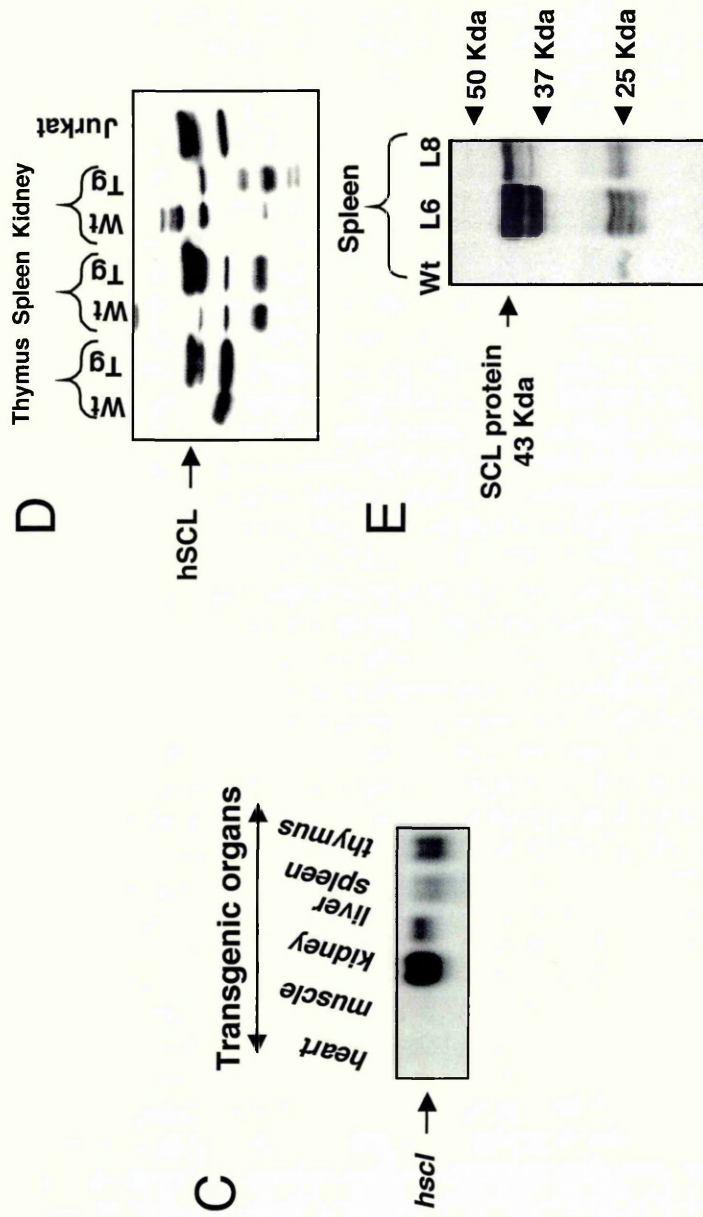


Figure 3.2

C. Expression of *hscL* mRNA in a transgenic L6 mouse. Northern blot analysis of 15 μ g of total RNA isolated from different tissues was hybridized with a *human scl* cDNA probe. The length of the transgene transcript is 1.2 Kb.

D. Expression of human SCL protein in high copy number L6 transgenic mice. Total protein extracts of different tissues of transgenic (Tg) or non transgenic mice (Wt) were separated on a 12 % SDS-PAGE, transferred to hybond-P PVDF membrane (Amersham) and probed with BTL-136 which recognizes the human and mouse scl protein. Nuclear extracts from Jurkat cells were used as a positive control. The 43 kDa hSCL protein is indicated by an arrow.

E. Level of SCL protein expression in animal lines L6, L8 and wild type littermates. 80 mg of total splenic protein extracts of the 10 copy L6, 2 copy L8 and wild type (Wt) animal lines were subjected to Western blot analysis using the human SCL protein specific antibody BTL-73 Mab. Quantification of hSCL protein shows that its level in L6 splenocytes is 5 times the level of L8 splenocytes. No SCL protein is detected in wild type controls.

animal line, the conclusions regarding the DNA-binding domain must be guarded, as possible integration effects cannot be ruled out. While the general physical appearance of the transgenic lines was normal, an obvious kinking and short length of the tail was observed in 100 % of the high copy L6 mice, 70 % of the two copy heterozygous L8 and 100 % of the homozygous four copy L8 mice (fig. 3.2B). Δb L3 transgenic mice did not have kinked tails. Taken together with the known expression pattern of the *Ly-6E.1* cassette in the Mullerian ducts and tail during embryogenesis, these results suggest that ectopic expression of hSCL induces female infertility, tail growth and bone abnormalities and that the severity of this phenotype is, in part, SCL dose- dependent.

3.2 Expression of *hscl* and $\Delta bhscl$ transgenes

As expected from previous analysis of the expression pattern of the *Ly-6E.1* cassette, *hscl* mRNA was present in thymus, spleen and kidney and not detected in the skeletal muscle and the heart (fig. 3.2C). In these organs, the mRNA expression pattern of *hscl* in the heterozygous L8 and of $\Delta bhscl$ in the Δb L3 transgenic mice was the same as in L6 animals, but the expression level was lower (data not shown). We next performed real time RT-PCR on total bone marrow of the 10 copy L6, the heterozygous 2 copy L8 and of wild type littermates. This assay revealed that the *hscl/hprt* mRNA ratio of the L6 animals was 6 times the expression level ratio of the L8 mice (Table 3.1). Thus, the transgene copy numbers in the two animal lines correlated well with their *hscl* mRNA expression level. In order to assess the transgene expression in different haematopoietic lineages we quantified *hscl* mRNA of FACS sorted bone marrow cells and thymocytes of L6 mice and their wild type littermate controls. Real time RT-PCR

Table 3.1 Quantification of *hscI* mRNA levels in different animal lines

| Animal lines | mean <i>hscI</i> mRNA | mean <i>hpvt</i> mRNA | mean <i>scl/hpvt</i> ratio |
|--------------|------------------------|------------------------|----------------------------|
| 10 copy L6 | 1.137×10^{-1} | 1.065×10^{-1} | 1.068 |
| 2 copy L8 | 8.144×10^{-3} | 4.572×10^{-2} | 0.178 |
| Wild type | 0 | 4.943×10^{-4} | 0 |

Real-time RT-PCR using Lightcycler technology (Roche) of unsorted bone marrow cells of L6, L8 and wild type animal lines was performed to show correlation between transgene copy number and mRNA expression. *ScI* mRNA was quantified relative to *hpvt* mRNA levels. All samples were run in duplicate and the mean *scl/hpvt* ratio of 2 experiments is shown.

Table 3.2 Effects of hSCL expression on peripheral blood cells

| Animal lines | B220+ | CD4+ | CD8+ | MAC-1+ | Sca-1+ Mac-1+ |
|--------------|------------|-----------|-----------|-----------|------------------|
| L6 | 19+/- 10.9 | 22+/- 3.4 | 26+/- 3.2 | 27+/- 4.5 | 19+/- 4.8 |
| Wild type | 48+/- 7.2 | 13+/- 1.8 | 11+/- 1.2 | 24+/- 4.5 | < 2+/-1 |

Peripheral blood cells from *hscI* L6 mice and their age and sex matched wild type controls were stained with anti-CD4 and anti-CD8 antibodies for T-lymphocytes, anti-B220 antibody for B lymphocytes and anti-Sca-1 and anti-Mac-1 antibodies for myeloid cells and analysed by FACS. Mean percentages and SD values of 6 experiments are shown and refer to the total white cell count. They do not exactly equal 100% because several stains were necessary.

showed a *hscl/hprt* mRNA ratio of 1 for B220 positive bone marrow cells and a ratio of 2 for the Mac positive population. The overall ratio in total thymocytes was 0.4 (data not shown). Real-time RT-PCR on DN, DP, CD4+ SP and CD8+ SP cells showed that the transgene was expressed in all thymocytes subsets in increasing order: DP, DN, SP cells. There was no significant difference in the mRNA expression level of CD4 and CD8 SP cells (data not shown). Hence, *hscl* mRNA expression was present in all the haematopoietic lineages studied and is in accordance with previously published data on the expression pattern of Ly-6E.1²⁵⁷. To examine whether the expression of hSCL protein was correlated with the expression of *hscl* mRNA, protein extracts from different organs were analysed by Western blot. A correlated expression between the *hscl* mRNA and the hSCL protein levels was found in all the positive organs tested, except for the kidney which expressed a high level of mRNA, but did not express the hSCL protein (fig. 3.2D). A comparative study of SCL protein expression was performed using spleen nuclear extracts of the 10 copy L6 and the 2 copy heterozygous L8 mice. The protein level detected in L6 animals was 5 times the level of L8 mice, thus showing the correlation between transgene copy numbers and protein expression level in the spleen (fig. 3.2E). A faint hSCL expression was found in the total bone marrow by Western blotting and immunohistochemical staining of bone marrow paraffin-embedded sections showed the presence of positive mononuclear cells in this organ (data not shown).

3.3 Peripheral blood FACS analysis of transgenic mice reveals abnormalities in circulating lymphocytes and myeloid cells

To determine whether ectopic expression of *hscl* or $\Delta bhscl$ under the control of the *Sca-1* regulatory sequence induces a defect in haematopoiesis, blood of 12 weeks

old transgenic and non-transgenic age and sex matched littermates was collected and subjected to full blood counts. This analysis showed an increase in the total number of white blood cells (WBC) in the transgenic versus non-transgenic mice ($3000/\text{mm}^3 \pm 200$ versus $2200/\text{mm}^3 \pm 200$, respectively) while the erythroid compartment, assessed by haemoglobin measurements and red blood cell numbers was not significantly altered (data not shown).

FACS analysis, performed on 12 weeks old L6 mice and their age and sex matched wild type controls revealed alterations of the peripheral blood cell distribution (Table 3.2). After gating on the total WBC, we found a 1.7 and 2.4 fold increase in CD4 and CD8 positive cells, respectively, leading to an alteration of the CD4/CD8 ratio of 1.2 vs 0.8 in non transgenic versus transgenic animals. Additionally, there was a 60% reduction in the absolute number of B cells identified by anti-B220 antibody. Interestingly, FACS analysis with anti-CD11b showed that the majority of L6 CD11b positive cells continued to express the cell surface marker Sca-1, whereas wild type CD11b positive cells were negative for this antigen in the peripheral blood (data not shown). All full blood counts and FACS analysis were performed on 6 transgenic and non-transgenic littermates.

Thus, abnormalities were only found in haematopoietic cells, which normally do not express SCL leading us to investigate haematopoietic and lymphopoietic organs.

3.4 The Sca-1 antigen expression persists on granulocytes and monocytes

After gating on the myeloid population, 100% of CD11b positive cells in the peripheral blood of L6 transgenic mice expressed the cell surface antigen Sca-1. In the bone marrow, we found a moderate increase of transgenic CD11b positive bone marrow cells. To define the mechanism underlying this moderate expansion

of the myeloid pool more closely, we performed methylcellulose cultures of total bone marrow and showed that the GM colony number was similar using transgenic or wild type bone marrow, while the size of the transgenic colonies appeared larger, thus indicating an increase in the proliferation potential of the transgenic granulo-macrophagic precursors (data not shown). Looking at the cell surface antigen, we found that, while only 5 % to 10 % of the CD11b⁺ cells expressed the Sca-1 antigen in the wild type mice and the low copy L8 line, more than 50 % of the CD11b⁺ cells were positive for Sca-1 in the high copy L6 and Δb L3 line (data not shown). As the bone marrow Sca-1 negative cells corresponded partly to macrophages (identified by the F4-80 specific antibody) and as the Sca-1 antigen is normally expressed on immature haematopoietic progenitors, the persistence of Sca-1 expression could indicate a block in granulocytic differentiation. This phenomenon has already been observed in granulopoietic cells that constitutively and inappropriately expressed SCL²²⁸. We therefore performed cytopspins of CD11b positive peripheral blood cells and of Sca-1/CD11b positive bone marrow cells and showed that their morphological stage of differentiation was similar to wild type CD11b positive cells (data not shown). Looking at the transgene expression using the BTL-73 anti SCL antibody we found that the transgene was extinguished in mature monocytes and granulocytes, indicating why we did not see a block in granulocytic differentiation.

3.5 Ectopic hSCL or ΔbhSCL expression under the control of the *sca-1* regulatory sequences alters thymic cellularity but does not lead to leukaemia

No T cell leukaemia developed in 150 transgenic mice during an observation period of one year. We first determined the type of cells that express hSCL protein in the thymus by performing immunohistochemical staining on paraffin-embedded

sections of this organ using a monoclonal antibody that recognised the hSCL protein and not the mSCL protein. A nuclear staining was found in most of the cells in the medulla and in a minority of cells in the cortex (fig. 3.3A). Examination of the thymus of wild type, L6 and Db L3 mice of different ages showed that the transgenic animals presented with a smaller thymus (fig 3.3B) and a decrease of absolute thymocytes numbers ($160 \times 10^6 \pm 10 \times 10^6$ versus $110 \times 10^6 \pm 10 \times 10^6$ at 4-8 weeks; $80 \times 10^6 \pm 10 \times 10^6$ versus $40 \times 10^6 \pm 10 \times 10^6$ at 12 weeks in wild type and transgenic mice, respectively). FACS analysis of the total thymic population did not show, in the younger animals, any significant alterations in thymocyte subset distribution. In contrast, 12 week old transgenic mice presented a 50% decrease of the DN cell population, a 30-50 % decrease in the absolute number of DP cells whereas the same absolute number of single positive (SP) CD4 or CD8 cells was observed in transgenic and non-transgenic mice (fig. 3.3C). Hence, taken together, these data indicate a relative increase in the number of SP cells with regards to the more immature cell populations. In order to investigate the causes for this relative increase further we performed FACS analysis on the CD8 SP cells which consist of two distinct type of cells: CD8 immature single positive cells (ISP) expressing low level of TCR- β and representing cells undergoing differentiation just before the double positive stage and CD8 mature single positive cells characterised by high level of TCR- β expression and representing differentiated cells. The expression level of TCR- β was analysed in CD8 SP cells, showing a 2 to 3 fold decrease of ISP cells in transgenic animals (data not shown) and indicating that the relative increase in the CD8 SP cells was not due to an increase in ISP cells.

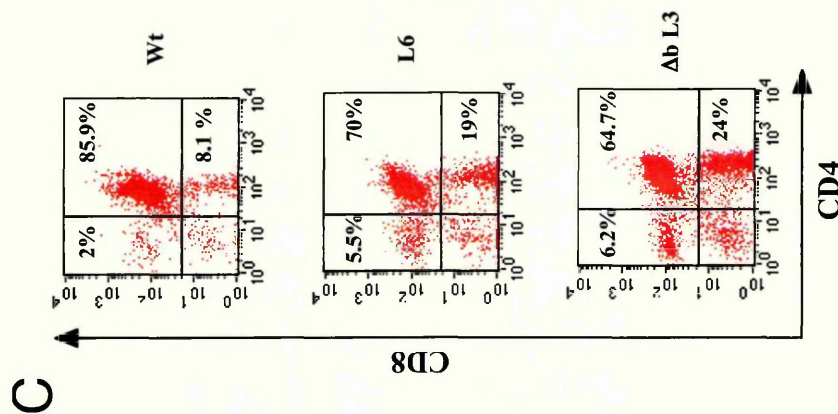
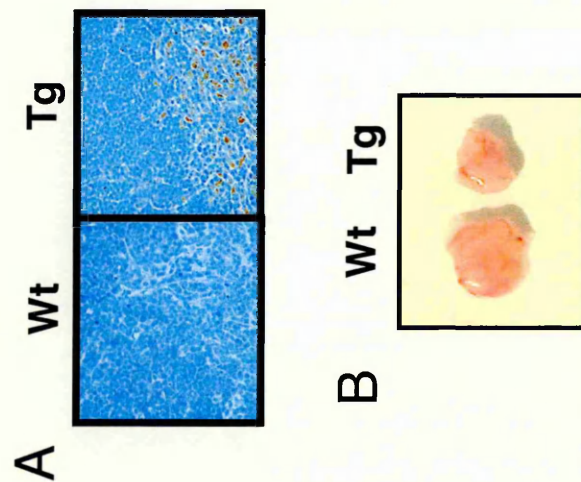


Figure 3.3 : Consequences of hSCL expression on thymus cellularity

A. hSCL protein expression in the thymus. Paraffin embedded thymus sections were stained with the BTL-73 monoclonal antibody which recognizes the hSCL protein but not the mSCL protein (original magnification: x120). **B.** Photo showing a representative wild type (Wt) and transgenic (Tg) thymus of two 4 week old sex matched littermates. **C.** Analysis of T cell subsets. Thymocytes freshly isolated from sex matched wild type, L6 and Δb L3 littermates at 12 weeks after birth were stained with anti-CD4-FITC and anti-CD8-PE antibodies. The percentages of marked cells are indicated in the quadrants. A representative experiment out of 6 is shown.

As signaling by the pre T-cell receptor (pre-T α) and T-cell receptor (TCR) regulates cell proliferation and differentiation of DN and DP thymocytes, we studied expression of these genes. Real-time RT-PCR analysis was performed on DN, DP, CD4⁺ SP and CD8⁺ SP cells purified from L6 transgenic mice and their wild type littermates. These experiments did not show any significant deregulation of *pT α* or *tcr α* gene expression in DN, DP or SP transgenic thymocytes (data not shown).

The absence of T cell leukaemia in transgenic mice may be related to low hSCL expression in transgenic T cells. Thus, we compared the hSCL expression level in Jurkat and L6 transgenic thymocytes by western blotting and showed that the hSCL protein level in Jurkat was at least 4 times higher compared to the L6 transgenic thymocytes (data not shown). Thus, assuming that the level of SCL expression in Jurkat cells represents the minimal threshold to cause tumors, *ly-6E.1* promoter weakness may be one reason why no T cell neoplasia occurred in our mouse model. Alternatively, other the variables such as mouse strain, transgene integration site or copy number have to be considered.

3.6 Ectopic hSCL expression leads to a significant reduction of early B-cell precursors

As blood analysis showed a significant reduction in B-lymphocytes, we performed FACS analysis on the lymphoid gate of the bone marrow of 12 weeks old L6 transgenic mice and age and sex matched littermate controls and showed a 40 to 50% reduction in the relative and absolute number of B220⁺ CD19⁺ cells. These

results were independent of the copy number and were also observed in the Δb L3 transgenic mice (fig. 3.4A).

To define the stage of a possible block in B cell differentiation, we performed multicolour fluorescent staining on the bone marrow of L6 and wild type animals using anti-B220, anti-CD19, anti-IL-7R α and anti-c-kit specific antibodies. B220⁺ CD19⁺ cells were divided into two distinct entities: one B220^{high} CD19⁺ population representing the more mature cells and one B220^{low} CD19⁺ subset consisting of immature B cells. Together with the IL-7R α antibody, we observed a 4 fold decrease of pre-B and late pro-B cells (B220^{low} IL-7R α ⁺ CD19⁺) in transgenic mice (fig. 3.4B). Additionally, using c-kit antibody to target the most immature B cell progenitors, we observed a three-fold reduction in the intermediate pro-B stage (B220^{low} IL-7R α ⁺ c-kit⁺) (fig. 3.4C).

To further characterise the defect in B cell lymphopoiesis, bone marrow cells of L6 and L8 transgenic and non-transgenic animals were subjected to *in vitro* pre-B colony formation assays. After 7 days of culture on methylcellulose we obtained 2 to 3 (L6, L8) and 12 (Wt) pre-B colonies, indicating that B cell differentiation was not only reduced *in vivo*, but also *in vitro* and suggesting an intrinsic defect within the lymphoid compartment. As fetal B cell development has been described to differ from the adult setting, we analysed the B cell population in days 16.5 to 18.5 fetal livers by FACS and found that, as in the adult bone marrow, the B cell compartment was affected by the ectopic expression of SCL protein at least up the pro-B cell stage (data not shown).

To analyse SCL effects on the expression of genes previously described as being important for B cell differentiation, we sorted B220⁺ CD19⁺ IL-7R α ⁺ (pro-B) and

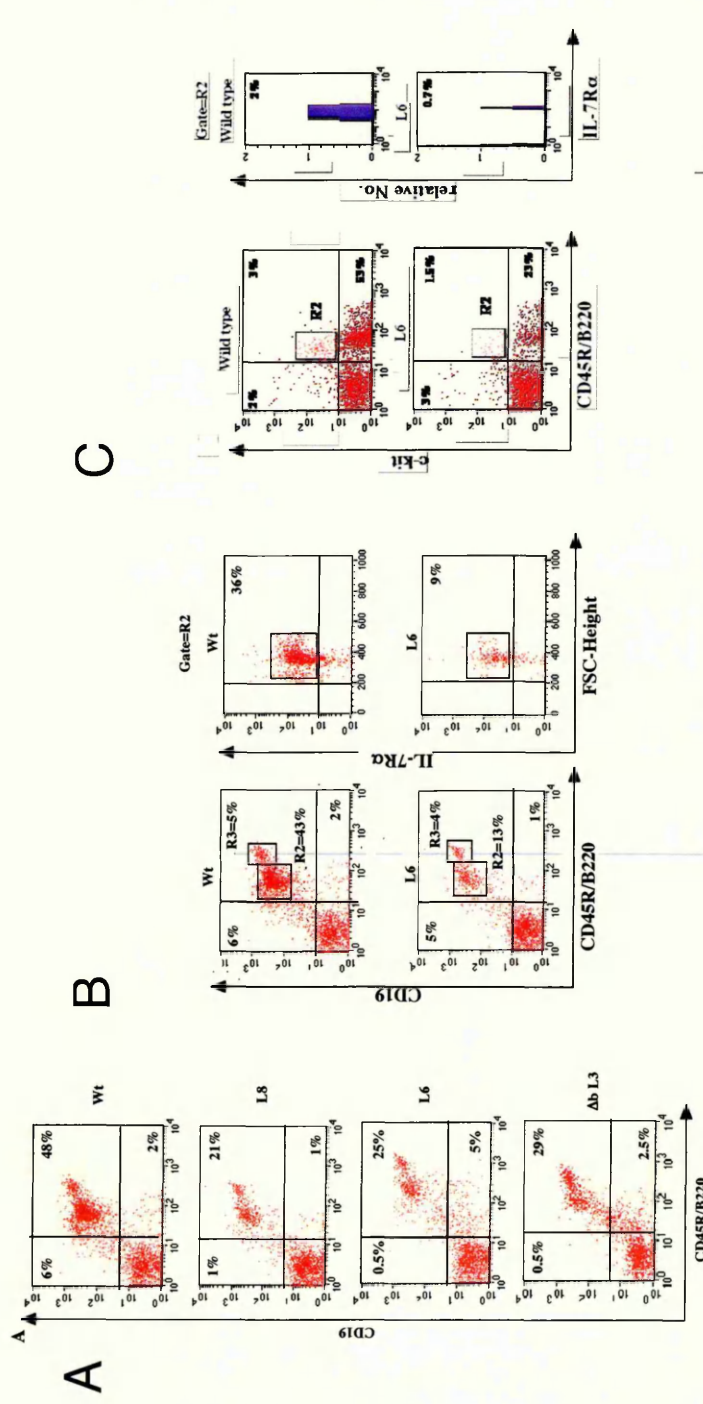


Figure 3.4: Defect in early B lymphopoiesis of transgenic mice
A. Phenotypic analysis of bone marrow B cells stained with anti-B220 FITC and anti-CD19 PE monoclonal antibodies. The percentages of marked cells are indicated in the quadrants.
B. Reduction of B220⁺ CD19⁺ IL-7Rα⁺ late pro B cells in transgenic mice. Cells were stained with monoclonal anti-B220-FITC, anti-CD19-PE and anti-IL-7Rα-biot/PE-Cy5. R2 represents the immature B220^{low} cells, R3 comprises more mature bone marrow B-cells. B220^{low} CD19⁺ cells in R2 were further analysed for FSC-Height and IL-7Rα expression.
C. Reduction of B220⁺ c-kit⁺ IL-7Rα⁺ intermediate pro B cells. Cells were stained with monoclonal anti-B220-FITC, anti-c-kit-APC and anti-IL-7Rα-biot/PE-Cy5. B220⁺ c-kit⁺ cells in R2 were further analysed in a histogram plotting anti-IL-7Rα fluorescence intensity against the relative number of IL-7Rα positive cells and shows the percentages of B220⁺ c-kit⁺ IL-7Rα⁺ cells in the lymphoid gate.
These FACS analysis were repeated at least 5 times and all percentages refer to the lymphoid gates defined on FSC-SSC.

B220⁺ CD19⁺ IL-7R α ⁻ (pre- B) cells from the bone marrow of transgenic and wild type mice and performed RT-PCR analysis. We demonstrate that, in cells escaping the defect in B cell differentiation, neither *pax.1*, *lef-1* nor *pu.1* genes were affected by hSCL expression in either of these cell populations (data not shown), implying that B-cell differentiation in these cells does not seem to be impaired.

3.7 Ectopic hSCL expression in B cells leads to a defect in isotype class switch recombination

We then studied the effects of ectopic hSCL expression on splenic B cell maturation. Morphology of the spleen of transgenic animals revealed a disorganisation of the germinal centre (fig. 3.5A). Absolute splenocyte numbers of transgenic and wild type littermate were similar and FACS analysis showed a 30 to 50% reduction of B220⁺ CD19⁺ and B220⁺ IgM⁺ cells in the spleen (fig. 3.5B and data not shown). In order to assess antigen dependent maturation further, we analysed the ability of transgenic splenocytes to undergo isotype class switch recombination upon activation with T cell independent antigen. As lipopolysaccharide (LPS) induces murine B cells to switch from IgM to IgG3 and IgG2b, we used LPS activation on sorted transgenic and non-transgenic B splenocytes. After exclusion of B220 negative cells, splenocytes derived from the high copy number transgenic mice showed a 4.5 fold reduction in IgG2b and IgG3 isotypes (fig. 3.5C and data not shown) after 72 hours, indicating an effect of ectopic hSCL expression on antigen dependent B cell differentiation. B220 positive splenocytes derived from the low copy number transgenic mice showed a reduction in IgG3 of only 1.9 fold thus implying that SCL induces an isotype switch recombination defect in a dose dependent manner (data not shown).

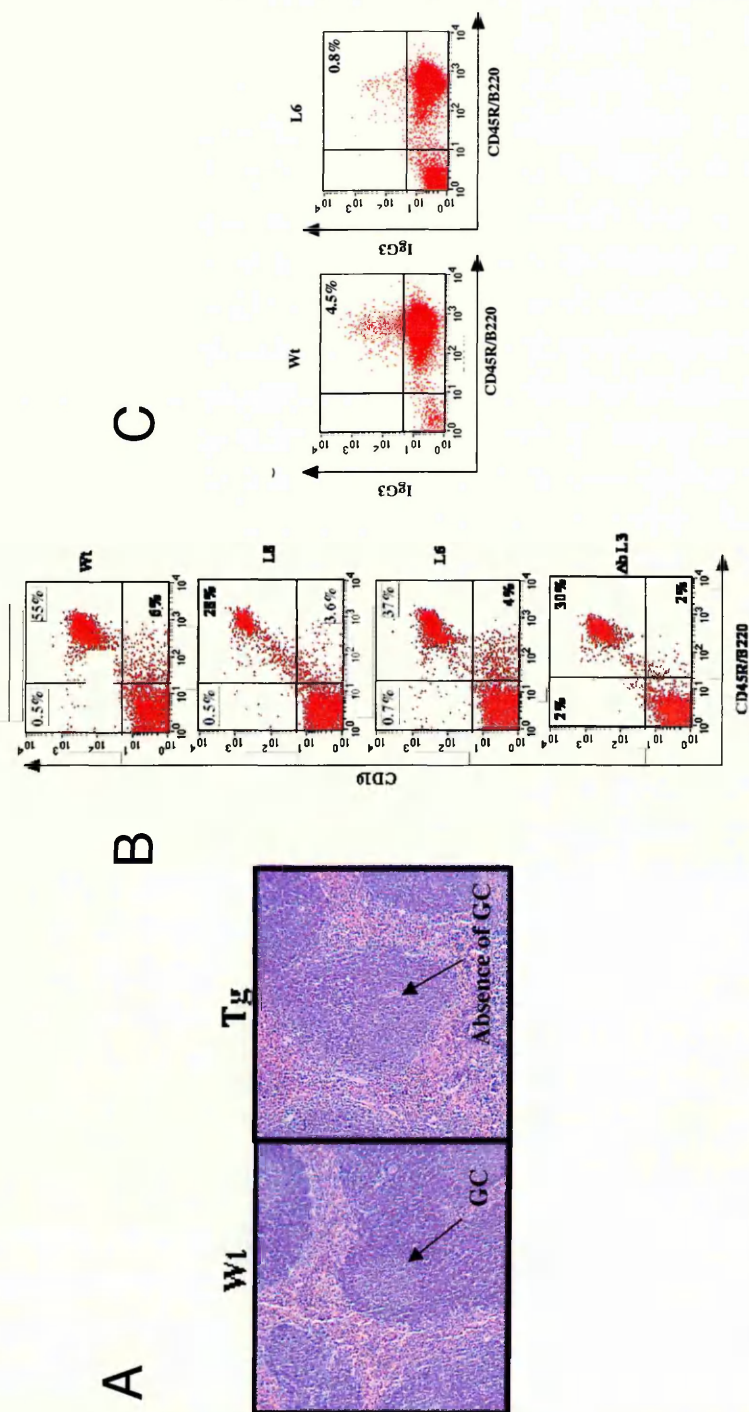


Figure 3.5: *Htal-1* transgenic mice show a reduction in splenocytes, disorganisation of the germinal centre and a defect in antigen dependent B cell maturation

A: Disorganisation of germinal centres (GC) in the spleen of transgenic mice. Morphology of paraffin-embedded sections of wild type and transgenic spleen revealed by haematoxylin eosin staining (original magnification : x120). **B:** Phenotypic analysis of B splenocytes of wild type, low copy (L8), high copy (L6) and $\Delta bL3$ transgenic mice. Cells were stained with monoclonal anti-B220-FITC and anti-CD19-PE antibodies. Percentages in quadrant refer to the lymphoid gate defined on FSC-SSC. Analysis were performed on sex and age matched mice and repeated 6 times.

C: Ectopic hTAL-1 expression causes a defect in immunoglobulin isotype switch recombination. Transgenic and wild type T-cell depleted splenocytes were activated with 25 μ g/ml of LPS for 3 days and then stained with monoclonal anti-B220-PE-Cy5 and anti-IgG3-FITC. Percentages of IgG3-positive cells are corrected after exclusion of B200⁺ cells.

As E2A is involved in class switch recombination and heterodimerizes with SCL we performed electrophoretic mobility shift assays (data not shown) on nuclear extracts of LPS activated B splenocytes of transgenic L6, heterozygous L8 and wild type littermates. This analysis showed specific binding of a SCL/E2A complex to *scf* oligonucleotides, which is supershifted by a hSCL specific antibody. The same complex, however much weaker, is present in splenocytes of L8 animals. Thus, a complex containing SCL/E2A is present in activated B splenocytes of transgenic mice and its expression correlates with the transgene copy number in each line. Together with previously published data^{228-230,279}, these results indicate that SCL protein may sequester E2A in these cells.

3.8 NOD/SCID mice transplanted with transgenic bone marrow display features of hSCL transgenic mice

B-cell differentiation has been shown to depend on the interaction of lymphoid precursor cells with the surrounding bone marrow stroma and microenvironment. We therefore aimed to exclude that the reduction in the B cell compartment was secondary to an extrinsic, cell non-autonomous phenomenon by performing transplantation experiments of transgenic and non-transgenic bone marrow into sublethally irradiated NOD/SCID mice.

At week 6 and 7 after transplant, blood was collected and FACS analysis performed. 75% of NOD/SCID mice injected either with transgenic or non-transgenic bone marrow, successfully engrafted. In NOD/SCID mice transplanted with transgenic bone marrow the Sca-1 antigen persisted on 30% to 50% of myeloid cells, indicating their transgenic origin. 11% versus less than 1% of peripheral blood lymphocytes of NOD/SCID mice transplanted with non transgenic

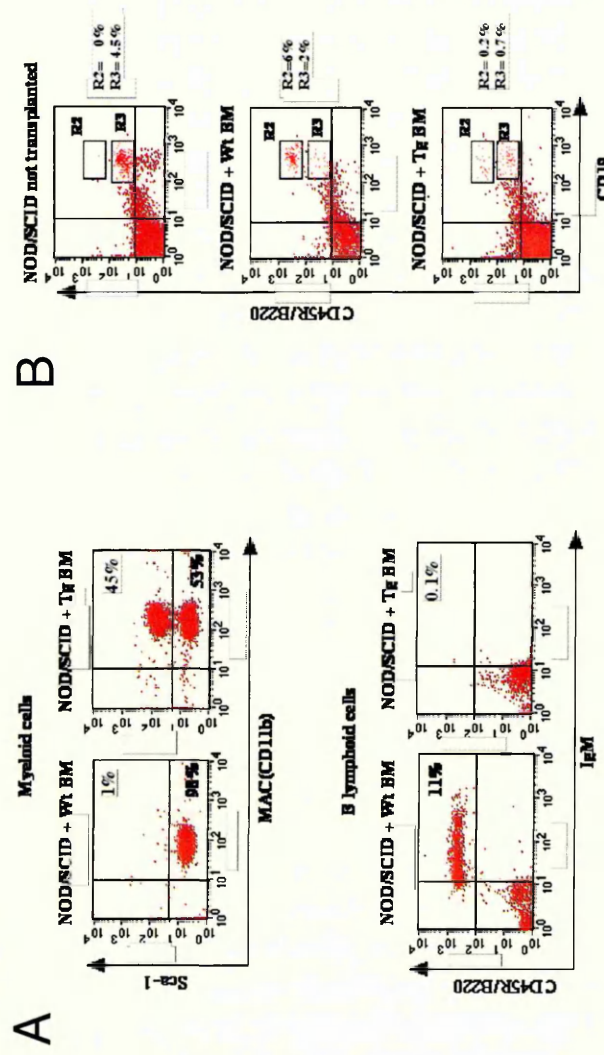


Figure 3.6: The reduction in the B cell compartment and the persistence of the Sca-1 antigen on myeloid cells due to ectopic SCL expression are cell autonomous phenomena

A. Peripheral blood cells of NOD/SCID mice transplanted with *hsc1* transgenic (Tg) or wild type bone marrow (BM) were analysed 6 weeks after transplantation for antigen expression using monoclonal anti-Sca-1-PE, anti-CD11b (MAC-1)-FITC, anti-B220-FITC and anti-IgM-PE antibodies. Transgenic CD11b⁺ cells show persistence of the Sca-1 antigen. NOD/SCID mice transplanted with *hsc1*⁻¹ transgenic bone marrow cells are deficient in B cells compared to mice transplanted with wild type cells. Percentages refer to myeloid and lymphoid gates, respectively.

B. Phenotypic analysis of bone marrow cells of NOD/SCID mice 8 weeks after transplantation. Cells were stained with monoclonal anti-B220-FITC and anti-CD19-PE. Percentages refer to the lymphoid gate defined by FSC-SSC. R2 comprises mature B cells, R3 the immature B220^{low} cells. Non-transplanted NOD/SCID mice normally show accumulation of B220^{low} CD19⁺ cells as shown in the first scatter. This population disappears in transplanted NOD/SCID mice and is replaced by donor cells.

versus transgenic bone marrow, respectively, were B220⁺ IgM⁺ cells (fig. 3.6A). Analysis of the bone marrow of all transplanted animals at 8 weeks post transplant showed that the population of B220^{high} CD19⁺ cells in transgenic grafts was severely reduced compared to wild type grafts. Non-transplanted NOD/SCID mice normally show an accumulation of pro B (B220^{low}) cells (fig. 3.6B, first scatter). These cells disappeared in transplanted NOD/SCID mice irrespectively of the transgenic or nontransgenic origin of the bone marrow as shown in the second and third scatter of fig 3.6B. This absence of pro B cell accumulation in the transgenic transplants indicates that a possible block in B cell differentiation may be prior to this stage.

Thus, this data shows that in contrast to nontransgenic transplants *hscI* grafts failed to re-establish early and late primary B cell lymphopoiesis and that the effects of ectopic hSCL expression on myelo- and lymphopoiesis are intrinsic to the cell populations and not secondary to an epiphenomenon of the transgenic microenvironment.

4 DISCUSSION

This work was designed to assess (1) the effects of ectopic hSCL expression on the lymphoid compartment and (2) to determine the importance of the DNA binding domain for some of SCL's functions. For these purposes, we generated two *hscI* transgenic models: L6 and L8 *Ly-6E.1-hscI* lines expressing different levels of the full length *hscI* transcription factor. Additionally, we examined the L3 *Ly-6E.1-ΔbhscI* line encoding for a mutant hSCL protein devoid of the basic domain which allows DNA binding. However, because we were able to obtain only one single animal line, statements regarding this domain must be made with caution.

Non-haematopoietic disorders

Transgenic mice presented haematopoietic and non-haematopoietic disorders. Apart from infertility, the most characteristic abnormality was the presence of kinked tails in 100% of high copy and 70% of low copy animals whereas they were absent in the $\Delta bhscI$ mutant suggesting that these phenomena were dose dependent and required hSCL DNA-binding. Dose dependency of bone related abnormalities and infertility has been observed previously in the *sil/scI* transgenic model²⁴⁸; in this model, the hSCL transactivation domain was not required to generate the phenotype. Our findings indicate that the DNA binding domain may be necessary to produce these ectopic non haematopoietic disorders and imply its potential importance for some SCL functions, although, due to reduced fertility of the mutant, we could investigate only one $\Delta bhscI$ animal line.

Transgene expression levels

Using real-time RT-PCR we showed a correlation between transgene copy numbers and mRNA expression level in the different animal lines. We could also establish a correlation between *hscI* mRNA and protein levels in different organs except for the kidneys which expressed high levels of transgene mRNA, but no protein. This discrepancy is in accordance with previously published observations on *lacZ* reporter gene expression under the control of Ly-6E.1 showing high mRNA but no protein expression in the kidneys²⁵⁷ and could be due to tight control of translation or protein degradation in this organ.

The stem cell pool is not affected

Despite its essential role in haematopoietic stem cells (HSCs), *Ly-6E.1-hscI* did not lead to major quantitative alterations of these cells neither under normal conditions nor after 5-fluorouracil treatment (data not shown). Furthermore, methylcellulose

colony forming assays did not show any significant difference in the number and morphology of colony forming units between transgenic and wild-type BM (data not shown). These results suggest that the expression level of hSCL in the *Ly-6E.1-hscl* transgenic HSCs might not be sufficient to increase the self-renewal potential of the HSC compartment or that the microenvironment may counterbalance hSCL action to ensure the homeostasis of the HSC pool.

Effects on the myeloid compartment

In contrast to previously published work^{212,230,245}, we did not observe a block in myeloid or monocytic differentiation, neither *in vivo* nor *in vitro*, in the transgenic mice. Instead, there was a moderate increase in myeloid cells and a subpopulation of transgenic CD11b (Mac-1) positive cells expressed the Sca-1 antigen; this phenotype was dose-dependent but DNA-binding independent indicating that its generation may need a threshold of hSCL expression in myeloid cells. This suggests that *sca-1* gene expression might be repressed during terminal myeloid differentiation and that the repressor might be sequestered by hSCL. Further investigations to address this question are necessary.

The defects observed in the lymphoid compartment may be due to sequestration of E proteins

T-cells

Importantly, ectopic hSCL did not induce T cell leukaemia in any of the transgenic lines in over 150 offspring during their first year of life. This is in contrast to data published on the single transgenic *lck/SCL* and on the double transgenic *SCL/ckIIα* or *SCL/Imo-2* and *SCL/Imo-1* mice^{244-246,248,249}. However, the onset of T-cell tumours in *lck/SCL* transgenic mice was characterised by low penetrance and long latency²⁴⁴. In contrast, *lck/SCL* mice with a p53 +/- background or double

transgenic mice developed the disease earlier and with a significantly higher penetrance. Thus, the ability of SCL to generate T-cell tumours in transgenic mouse models is likely to depend not only on the regulatory sequences used to mediate SCL expression in mice and on variables such as mouse strain, transgene integration site and copy number, but also on additional oncogenic events or co-expression of other oncogenic transgenes.

In our model, we observed a decrease in the number of immature DN, ISP CD8⁺ cells and DP subsets which was not due to increased apoptosis (data not shown). However, compared to non transgenic mice, the number of mature SP cells remained unchanged indicating an increased differentiation capacity from the DP to the SP cell stage or a proliferation ability of the transgenic SP cells. T cell-specific E box complexes are largely composed of E2A/HEB heterodimers; however, their DNA-binding activity is decreased upon transition from the DP to the SP cell subset^{111,280}. Thus, ectopic expression of SCL could perturb this balance and induce an increase in the differentiation rate of the DP cells. A recent study using standard RT-PCR followed by PhosphorImaging quantification showed a significant reduction in *pTα* expression in DN cells and in *tcrα* expression in the DP population in *scl/lmo1* transgenic mice which went on to develop leukaemia¹⁴⁹. Using real-time PCR we could not detect a down-regulation of these genes in DN, DP or SP cells of *hscl* single transgenic mice thus indicating that the decrease in DP cells is not due to a deregulation of these genes. Besides, we did not detect *lmo2* mRNA in DP nor SP thymocytes (data not shown). Thus, our results suggest that ectopic SCL expression during T-lymphopoiesis alters cell proliferation and differentiation via *pTα* and *tcrα* gene expression independent mechanism and may

represent a state in which additional events like LMO-1/2 expression are necessary to lead to the leukaemic phenotype.

B-cells

The most striking characteristic of *Ly6E.1-hscI* and *Ly6E.1-ΔbhscI* transgenic mice was their reduction in B cells. This phenotype has not been described so far in neither *in vivo* nor *in vitro* studies and is certainly due to a specific SCL activity as, in contrast to our results, transgenic mice generated using the *Ly-6E.1-bcl2* cassette present an increase of the B cell compartment in spleen and bone marrow (unpublished data, Dzierzak E.). Although SCL has not been directly implicated in normal or malignant B-cell biology, bHLH dosage plays a critical role in B-cell differentiation. Because SCL is able to heterodimerize with E2A *in vitro*, we further investigated its effects on B-cell development. The *hscI* transgene induced a reduction in the fetal and adult B cell compartment including the most immature stages. However, antigen independent maturation of B cells escaping this effect seemed to be normal. Finally, by transplanting transgenic bone marrow cells into NOD/SCID mice we showed that the B cell defect induced by SCL was cell autonomous.

The B cell deficiency observed may be due to a differentiation block, similar to the one described in various gene inactivation studies such as *Pax-5^{-/-}* knock out mice²⁸⁰. As *pax-5* has recently been described as a target gene for E2A²⁸¹, titration of E2A by hSCL in pro and pre B cells should alter *pax-5* gene expression. However, in pro and pre B cells escaping the defect in B cell differentiation, we did not detect any alteration in *pax-5* gene expression, implying that SCL does at least not completely sequester E2A in pro and pre B cells. Additionally, in contrast to the *Pax-5^{-/-}* knock out experiments, we could not detect an accumulation of immature

pro B cells. This may suggest that if there is indeed a block in differentiation it must be early before the pro B cell stage. The effect observed occurred at low and high copy transgene numbers, implying that even low levels of SCL expression are sufficient to perturb the transcriptional balance of B-lymphopoiesis. In addition, the same B-cell defect was also observed in the Δb mutant, supporting the idea that hSCL may exert transcriptional activity via a DNA binding independent mechanism by forming part of a large multi protein complex. This is consistent with previous data on hSCL acting as a co-factor of GATA-3 in a complex containing LMO2²⁵⁴ and particularly with the rescue of primitive and definitive haematopoiesis by Δb SCL¹⁸⁸.

We also found disorganisation of the germinal centre and defect in isotype class switch recombination of mature B cells. Similar morphological features have been found in mice lacking OBF-1, a transcription factor involved in class switching²⁸². As E2A is required in class switch recombination²⁷⁶ we performed EMSA on nuclear extracts of LPS activated B splenocytes and showed that the heterodimer SCL/E2A was present in these cells and that its level correlated with the transgene copy number. Additionally, the class switch recombination defect observed was dependent on the expression level of SCL protein indicating that it is most likely due to titration of E2A. This is in contrast to the reduction in the B cell compartment, which was dose-independent and thus suggests other possible mechanisms of SCL action on B cell differentiation. Taken together, ectopic SCL expression sheds further light on the critical role of E-proteins in B-cell development.

In conclusion, we have shown that overexpression of hSCL alone in a transgenic scenario does not only lead to an imbalance in thymocyte maturation but also to a

most unexpected block in B lineage differentiation. Here, we have indicated both DNA-binding independent and dependent functions of SCL and show that the *SCL* gene extinction observed during myelopoiesis and B and T lymphopoiesis is necessary for the normal development of these haematopoietic lineages.

5 ANNEX TO DISCUSSION

This first project allowed me to become familiar with some of the important techniques used in molecular and cellular biology. However, it had severe limitations directly linked to the transgenic approach used. With hindsight, it seems to me that an over-expression model using a non-inducible system may not be the ideal approach to further define the role of SCL in haematopoiesis and leukaemia. Besides, the detailed characterisation of its effects on B cells, where SCL is not normally expressed, is questionable and the results obtained simply strengthen the importance of E proteins in B cell development.

I have come to the following five main conclusions:

1. The Sca-1 cassette is not suitable to drive expression of SCL in the HSC compartment. This may be due to insufficient levels of transgene expression in each cell. Besides, the mouse strain used expresses Sca-1 in only 25% of stem cells with repopulating activity. If these cells have a survival disadvantage, they may be substituted by HSC that do not express the transgene. Moreover, recent evidence from conditional knockout studies suggests that SCL is dispensable for normal function and maintenance of adult HSC⁷⁸. Thus, it may not be surprising that the SCL transgenic mice did not show any abnormalities at the HSC level.
-

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2. In cells that do not normally express SCL, such as lymphocytes, over-expression of SCL leads to a profound disturbance of the normal differentiation program probably via interaction with important regulators of lymphopoiesis like the E proteins. This is of relevance in T cells, where ectopic SCL expression is seen in leukaemia.
 3. Under the control of the Sca-1 promoter, SCL is expressed in T- cell progenitors. However, this experiment does not lead to T cell leukaemia. Therefore, either levels and/or the precise timing of SCL expression in T-cell development are critical for leukaemogenesis.
 4. Additional oncogenic events are likely to be required to enhance the leukaemic potential of SCL and it is thus likely that SCL is involved in a molecular cross talk with other nuclear proteins.
 5. Mice expressing the SCL DNA-binding mutant present with the same pre-leukaemic phenotype, further strengthening the hypothesis that, in lymphopoiesis, as in stem cell specification, SCL does not act through direct DNA-binding. Its function is therefore very likely to rely on protein-protein interactions through its HLH domain.

Therefore, a logical progression of this work was to try and identify protein partners of SCL .

CHAPTER IV: ATTEMPTS TO IDENTIFY SCL-CONTAINING COMPLEXES BY CONVENTIONAL IMMUNOAFFINITY

1 INTRODUCTION

To date, a number of laboratories have attempted to identify additional partners of SCL in haematopoiesis and leukaemogenesis by using expression cloning strategies but have not been able to characterise other informative partner proteins in addition to those already known. To begin my search for SCL partners we opted for the Far Western approach, a technique which had been successfully used, for example, to identify co-activators of nuclear hormone-receptors²⁸³. I made and screened a cDNA expression library from mRNAs extracted from a patient with a T-ALL carrying a *sil* deletion. As SCL is essential for erythromegakaryocytic differentiation (see chapter I) I also screened human megakaryocyte and erythroid protein expression libraries. Unfortunately, none of these screens were informative because of high non-specific background. Besides, this technique only detects binary interactions. As SCL always functions as a heterodimer, this strategy was certainly not the most appropriate to identify additional partners. We therefore decided to adopt a different approach and chose immunopurification of SCL-containing multiprotein complexes with subsequent mass spectrometric analysis. In the following paragraph, I wish to delineate some of the technical considerations associated with this approach.

1.1 Immunopurification

The successful outcome of conventional immunopurification (IP) depends on the availability of specific high affinity antibodies.

The IP is commonly preceded by several steps of prepurification to reduce the complexity of protein lysates or nuclear extracts. This consists of an antigen concentration step followed by size fractionation of the protein extract and/or purification by ion exchange and subsequent IP. The first two steps are necessary to increase amount and concentration of the antigen in the input, the IP itself adds specificity. The IP step is performed in stringency conditions which preserve protein-protein interactions in multimeric complexes. The antigen-antibody complexes (and proteins associated to the antigen) are pulled-down with Sepharose beads coated with protein A or G. Affinity of protein A or G to the Fc fragment of antibodies is species - and IgG isotype-dependent. For example, protein A strongly binds to polyclonal rabbit IgG, but only weakly to murine IgG1 and rat IgG2a and 2b whereas Protein G binds all IgGs except for chicken. It is important to be aware of the different characteristics of available antibodies. As a general rule, polyclonal antibodies have the advantage of high affinity to both antigen and sepharose beads. However, specificity is usually inferior to monoclonal antibodies. Monoclonal antibodies on the other hand can be very specific, but their affinity to both antigen and sepharose beads is often low.

Affinity to beads can be increased by using bridging antibodies between the antibody used in the IP and the beads. In order to prevent IgG heavy and light chain shredding, it is common practice to crosslink antibodies to beads prior to mass spectrometric analysis.

1.2 Mass spectrometry

Traditionally, mass spectrometry (MS) has been regarded as an indispensable yet expensive, complicated and time consuming tool in protein chemistry. However, in recent years, due to significant improvements of the technology and its ability to

Q-TOF Mass spectrometry

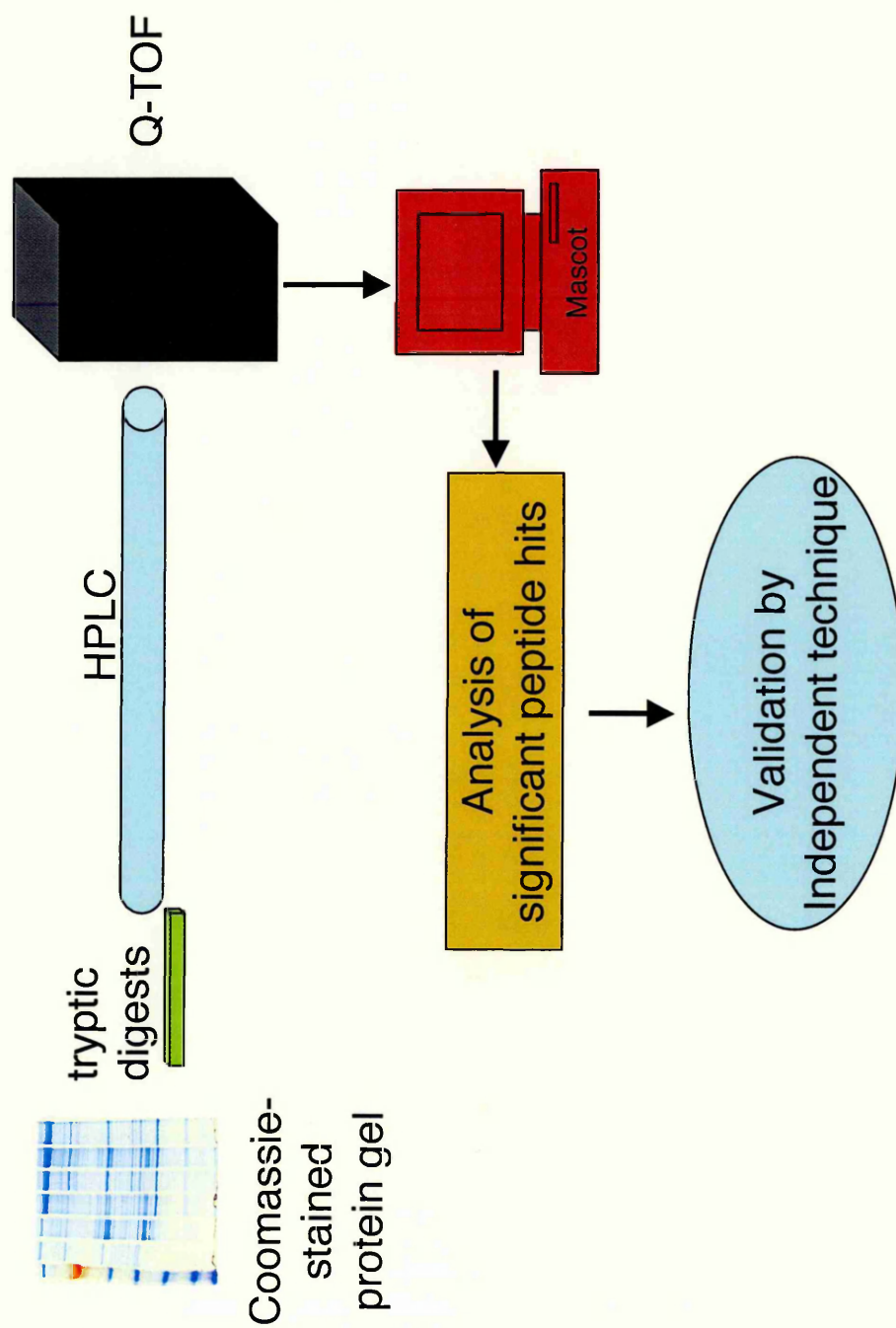


Figure 4.1 Mass spectrometry

identify proteins in complex mixtures, it has become of increasing value for life sciences and medicine.

By forming gas-phase ions from intact, neutral molecules, MS analysis can provide the accurate molecular weight of proteins and peptides with masses up to 500 kD using only a few picomoles of material and with an accuracy of around 0.01% of the calculated mass. In comparison, SDS-PAGE have accuracies of only 5-10% ; moreover, the mobility of the protein in the gel can be grossly affected (up to 50%) by the presence of covalent modifications such as lipids and carbohydrates. Because of this accuracy, a difference between the observed and the calculated mass of a protein analysed by MS can give invaluable clues as to the structure of the protein in question.

Besides, MS also has the ability to provide amino acid (AA) sequence information on peptides using a methodology called tandem MS (MS/MS). With MS/MS, sequences of up to 25 AA can be identified.

All mass spectrometers have three essential components : an ion source, the mass analyzer, and a detector. Ions are produced from the sample in the ion source using a specific ionization method. The ions are separated in the mass analyzer based on their mass-to-charge (m/z) ratios and then detected by an electron multiplier. The data system produces a mass spectrum, which is a plot of ion abundance versus m/z . MS/MS consists of two stages : the first stage serves to isolate a peptide precursor ion of interest based on its m/z , and the second stage to mass analyse the product ions formed by spontaneous or induced fragmentation of the selected precursor ion. Interpretation of the product-ion spectrum provides sequence information for the peptide selected.

Within a few years of their introduction in 1988, two different ionization methods called electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI)^{284,285} had supplanted previous techniques. By cocrystalizing analyte molecules with an excess of an UV absorbing matrix, MALDI-MS produces singly charged peptide ions which are then analysed by a time-of-flight (TOF) analyzer. By contrast, in ESI, multiply charged ions are formed from peptides by spraying a dilute solution of the analyte into the atmosphere chamber before analysing them in a triple quadrupole mass filter (for review^{286,287}).

The sensitivity of both methods is in the low-picomole range. ESI-triple quadrupole analyses molecules within 150 kD, MALDI-TOF achieves information on molecular weights of over 350kD. However, in the molecular weight range from 5-50 kD, ESI is much more accurate and provides better mass resolution. Generally, MALDI is less sensitive to contamination with buffers and salts, but intolerant of SDS. However, with the advent of high performance liquid chromatography (HPLC) combined to ESI (LC-ESI) and new desalting methods, the problem of sample contamination with ESI has been alleviated. In addition, LC-ESI allows the separation of complex protein mixtures prior to MS analysis whereas MALDI requires simple protein samples. LC-ESI however, takes about 70 minutes per sample and only one sample can be analysed at the time. In contrast, MALDI allows high through-put analysis. This implies that MALDI is the ideal tool when dealing with protein spots from two dimensional gels or with protein-protein interaction arrays and antibody coated arrays. LC-ESI on the other hand is used for the identification of proteins after one dimensional separation techniques.

The more widespread need of mass spectrometry analysis in life sciences and medicine has triggered the development of several new techniques and analysers.

Importantly, the analyser in our study, called Q-TOF, is increasingly used to analyse complex protein mixtures (figure 4.1) (for review²⁸⁸). It consists of a hybrid between LC-ESI-triple quadrupole analyser and MALDI-time-of flight using HPLC-ESI or MALDI combined with a quadrupole **and** time-of-flight mass analyser (Q-TOF).

Besides, in the context of medical research and diagnostics, it is worth mentioning that the introduction of novel technologies such as surface-enhanced laser desorption/ionization MALDI already permits high through-put analysis of crude serum or urine samples. Finally, imaging MALDI-MS has been used to analyse proteins of tissue sections which were thaw-mounted on a target plate and coated with matrix (for review²⁸⁹).

2 OUTLINE OF EXPERIMENTS

A general outline of the conventional multi-step purification strategy is given in figure 4.2A. We decided to adopt this approach after initial unsuccessful attempts to immunopurify SCL in a single step from crude nuclear extracts or from extracts prepurified by weak anionexchange. Briefly, MEL nuclear extracts were prepared from 4 litres of cell culture. 25mgs were precipitated using 55% Ammoniumsulphate and the resuspended precipitate containing SCL protein was loaded on a Superose 6 H/R sizing column. Gel filtration fractions were screened by Western Blot for SCL and its known partners and SCL-containing fractions were used as the input for immunopurification. A polyclonal rabbit antiserum raised against the C-terminal (Ct) domain of SCL¹⁸⁸ was affinity-purified and used in the IP reaction.

3 RESULTS

3.1 Size fractionation reveals that SCL co-migrates in high-molecular weight fractions with known partners

In vitro analyses suggest that SCL is part of multiprotein complexes in erythroid cells and *in vivo* studies show co-occupancy of regulatory sequences of haematopoietic-specific genes by SCL and members of the pentameric complex (see Chapter I). To show that, *in vivo*, SCL can form multimeric protein complexes in erythroid cells, gel filtration analyses of nuclear extracts prepared from uninduced mouse erythroleukemic (MEL) cells were performed. Upon fractionation of the nuclear proteins on Superose 6 columns, collected fractions were subjected to Western Blot analysis (figure 4.2B). This revealed that SCL, as well as two previously characterised partners GATA-1 and Ldb1, co-elute in fractions corresponding to molecular weights of 670 kDa and above (fractions 6 to 10) thereby indicating that they are part of high molecular weight multiprotein complexes. A small proportion of SCL was also detected in a fraction corresponding to lower molecular weights (fraction 18). Very similar results were obtained after fractionation of nuclear extracts purified from a megakaryoblastic cell line (L8057, data not shown). Therefore, in erythroid cells and megakaryocytes, SCL and its partners GATA-1 and LDB-1, are very likely to be associated with nuclear proteins to form one or more high molecular weight complexes. A fraction of SCL may also exist as heterodimers with E-proteins or in small protein complexes, unless detection of SCL in fraction 18 is the result of the dissociation of the high molecular weight multiprotein complexes during the purification and fractionation processes.

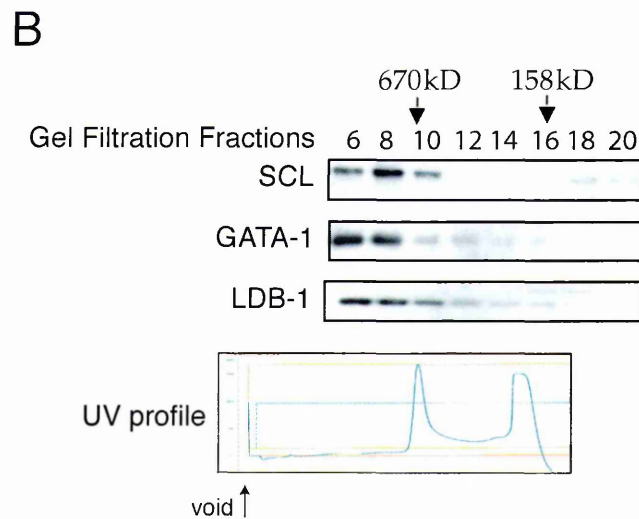
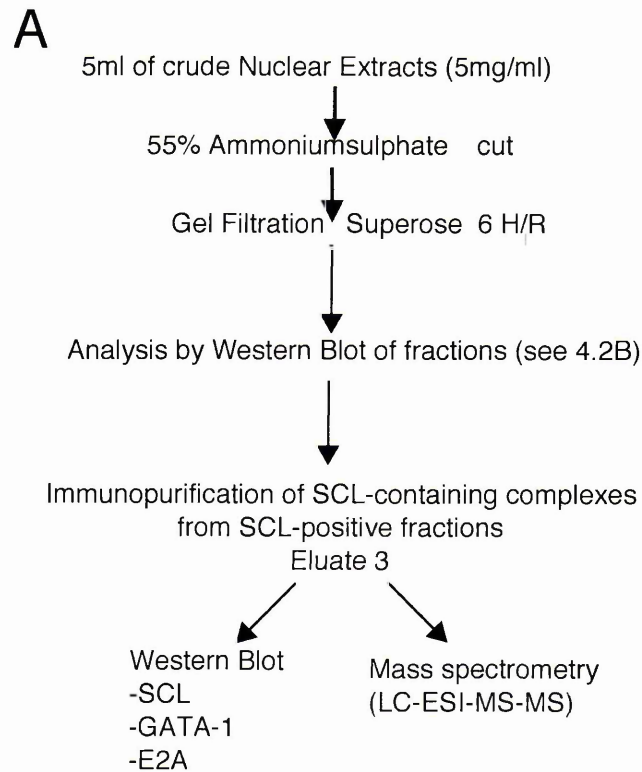


Figure 4.2

A Purification of SCL-containing multiprotein complexes: the immunopurification strategy

B Gel-filtration analysis

Nuclear extracts were prepared from MEL cells, fractionated by a Superose 6 H/R gel-filtration column and analysed by Western blotting.

The numbers over the lanes represent the eluted fraction numbers, and protein molecular masses are indicated by arrows.

3.2 P107 may be a partner of SCL

This initial pre-purification step allowed to reduce the complexity of the nuclear extracts to a great extent. Gel filtration fraction 8 was then chosen to perform pull-down experiments using immunopurified rabbit α SCL antibodies crosslinked to Protein G beads. Antigen was eluted from the beads with 100 mM glycine pH 2.5 in six fractions. These fractions were then tested by Western Blot for presence of SCL. SCL and the known partners GATA-1 and E2A co-eluted in fraction three (figure 4.3A), thus showing that known SCL partners had been co-purified and, therefore, validating the technique. A typical profile of the SCL-positive eluate 3 from MEL and L8057 nuclear extracts is presented in figure 4.3A after separation on a 4-12% SDS-Page gel and silverstaining. It reveals a number of discrete bands which are present in the IP lane with α SCL, but not in the negative control with rabbit polyclonal immunoglobulin (IgG). To identify additional partners of SCL, the precipitated proteins were subjected to mass spectrometry analysis. For this, silverstained bands were cut out and the proteins digested with trypsin. However, mass spectrometry analysis proved very difficult because of the weakness of some of the bands and because of structural modifications of the proteins caused by the silver staining process despite immediate destaining steps. To circumvent these problems, we scaled up the immunopurification, separated the eluate by SDS-PAGE and subsequently stained with Sypro Ruby gel stain. This fluorescent staining method does not interfere with subsequent mass spectrometry analysis and, as a general rule, it is less sensitive than silver-, but more sensitive than and colloidal Coomassie-staining. From MEL pull-downs, we visualized one band at around 100kD (data not shown) which was identified by mass spectrometry as

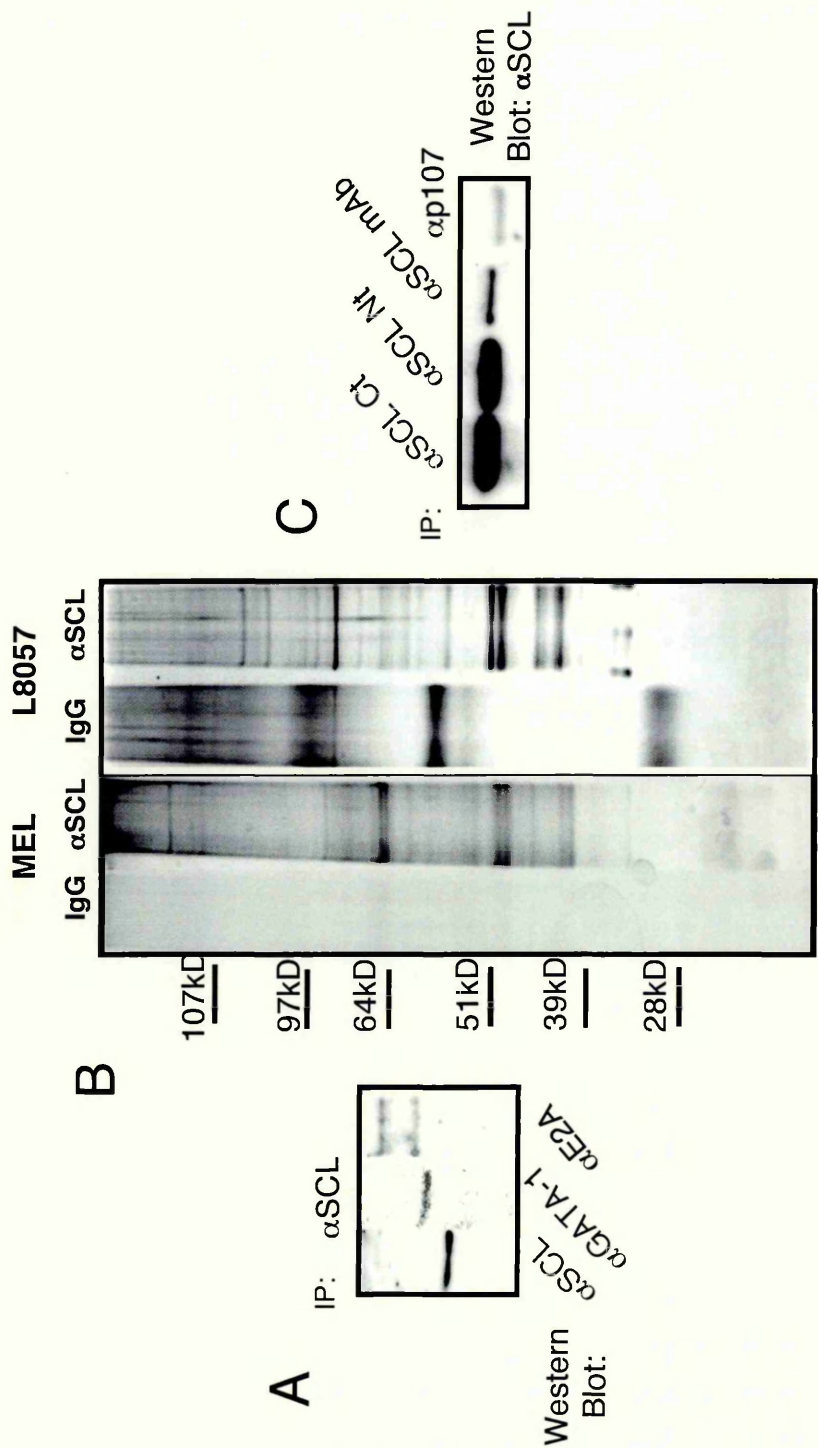


Figure 4.3 Gel fractionated nuclear extracts derived from MEL or L8057 cells were immunopurified with α SCL Ab (IgG= negative control). Immunoprecipitated products were eluted from the beads. Elution fraction 3 was loaded on 4-12% SDS-PAGE for subsequent analysis by Western Blot for presence of SCL, GATA-1 and E2A (figure 4.3A) or silver-staining (figure 4.3B). **A** Western Blot analysis of eluate 3 of the purification revealed the presence of SCL and two of its partners, GATA-1 and E2A. **B** Silverstained gradient gel of elution fraction 3. **C.** Co-immunoprecipitation from MEL nuclear extracts using antibodies indicated on top of the figure. Note, that α p107 antibodies immunoprecipitate SCL, thereby validating the mass spectrometry results.

p107, a retinoblastoma related protein. As retinoblastoma protein has been described as a potential partner of SCL in MEL cells²²⁶ we attempted to validate this result by reverse co-immunoprecipitation from MEL nuclear extracts using polyclonal rabbit α p107 antibodies. The results of the Western Blot with anti-SCL are illustrated in Figure 4.3C: p107 is able to pull down a small fraction of SCL and is therefore likely to be a partner of SCL.

4 DISCUSSION

This conventional immunopurification procedure was a first step towards the purification of SCL-containing multi-protein complexes.

1) The gel filtration data presented shows for the first time that SCL and its partners are part of high molecular weight complexes *in vivo*. 2) We were able to validate the purification procedure by pulling down known partners of SCL from gel filtration fractions. 3) After scaling-up the purification procedure and changing the staining technique, I could identify p107 as one potential partner of SCL in MEL cells.

P107 is a member of a family of three closely related mammalian proteins which includes p130 and the widely known tumour suppressor retinoblastoma protein (RB) (for review²⁹⁰). Together, these proteins are known as the pocket proteins because their main sequence similarity resides in a domain (the pocket domain) that mediates interactions with E2Fs and other factors including viral oncoproteins. Overexpression experiments have indicated functional similarities between the three family members in the regulation of the cell cycle. Thus, all of them cause arrest in the G₁ phase of the cell cycle by interacting with and repressing E2F-

mediated gene transcription. In addition, they are all phosphorylated by cyclin-dependent kinases (CDKs). Genetic experiments suggest that the three proteins have partially redundant as well as opposing functions (for review²⁹¹).

The most extensively studied member of this family is RB, the first tumour-suppressor gene ever to be cloned^{292,293}. RB is known for its involvement in familial cases of retinoblastoma. In the haematopoietic system, it is expressed in multipotent progenitor cells and throughout erythroid differentiation²⁹⁴. Rb-/- mice die between 13 and 15 dpc with a pronounced defect in erythroid and neuronal development and both intrinsic and extrinsic functions have been put forward to explain the haematopoietic defects observed in Rb-/- mice^{295,296}. Interestingly, it has been shown in transactivation experiments that RB represses *c-kit* gene activation upon interaction with the SCL-containing pentameric complex in erythroblasts²²⁶. It seems that the repressive action of RB on regulating gene expression occurs, at least in part, via the recruitment of chromatin remodelling complexes to promoter regions. These complexes mediate chromatin condensation and subsequent inhibition of transcription^{297,298}.

The role of the retinoblastoma related protein p107 in haematopoiesis is less clear in that the phenotype of the p107 single knock-out is mouse strain-dependent and does not have haematopoietic abnormalities. However, p107 recruits repressor complexes containing HDACs to E2F promoter regions similar to retinoblastoma protein^{299,300}. Thus, together with these published data, our results indicate that SCL may act as a repressor in erythroblasts via interaction with members of the pocket family of retinoblastoma related proteins.

In conclusion, we have described a potentially interesting interaction between SCL and p107. However, as discussed, there were clear technical limitations associated

with the immunoaffinity purification. We therefore decided to opt for a different approach to identify SCL-containing protein complexes.

CHAPTER V: THE BIOTIN-STREPTAVIDIN SYSTEM

1 INTRODUCTION

1.1 The biotin-streptavidin purification strategy

The biotin-streptavidin purification approach I used was developed in collaboration with John Strouboulis²⁶². It involves stable expression of the protein of interest (SCL) tagged with a 23 amino acid sequence that can be efficiently biotinylated *in vivo*, in cells that also stably express the Escherichia Coli BirA biotin ligase protein. Biotin is a naturally occurring cofactor of metabolic enzymes, which is active only when covalently attached to the enzymes through the action of specific protein-biotin ligases. Biotinylated substrates can be bound very tightly by the proteins avidin and streptavidin. This is a strong noncovalent interaction, several orders more avid than that of any other commonly used affinity tags and immunoaffinity methods. As such, this system offers advantages over conventional purification techniques. First, purification of the biotinylated protein is performed under more stringent conditions, thus reducing background binding often observed with other affinity tags. Second, as there are relatively few naturally biotinylated proteins, purification of false-positive partners is reduced compared to IP procedures using antibodies which may cross-react with many other unrelated proteins. The 23 aminoacid tag was selected through multiple rounds of screening combinatorial peptide libraries for specific biotinylation by BirA biotin ligase³⁰¹. Such tags do not interfere with normal protein function and localisation, and are biotinylated *in vitro* with kinetics similar to those of a natural biotin acceptor³⁰². As such, it has been demonstrated that these tags serve as excellent substrates for efficient biotinylation in cells by co-expressed biotin-ligase.

2 OUTLINE OF EXPERIMENTS

To set up the technique, the mouse erythroid (MEL) and megakaryoblastic (L8057) cell lines were first transfected with the biotin ligase BirA. Stable clones, expressing high levels of BirA cDNA under the control of the strong ubiquitous human EF-1 α promoter were identified by Northern Blot (see figure 5.1 for general outline of experiments and figure 5.2A for Bir A constructs). They were subsequently transfected with an SCL cDNA tagged with a sequence coding for 23AA that can be biotinylated *in vivo*, and expressed under the control of the EF1- α promoter (figure 5.2B). MEL and L8057 clones expressing levels of biotinylated SCL similar to that of endogenous SCL in wt cells were selected. Crude nuclear extracts were prepared from 4 litres of cell culture (Dignam procedure) and purification of SCL-containing complexes performed using streptavidin beads. Complexes were eluted from the beads and separated by SDS- PAGE electrophoresis. Precipitated products were then analysed by Western Blot and mass spectrometry.

In parallel, I also set up the system in SCL-/- ES cells in order to be able to show in rescue assays that biotinylated SCL retained its functions in haematopoietic cells.

2.1 The SCL mutants

In addition, we used this experimental procedure to test the capacity of chosen SCL mutants to bind to protein partners. There were two aims to this : 1) to identify partners of SCL interacting with residues within the bHLH domain crucial for SCL function ; 2) to characterise the domains and residues of SCL mediating the interaction with newly identified partners. These mutants have been previously characterised in detailed structure-function studies^{188,193} (see chapter I) and are summarized in figure 5.3. Briefly, they consisted of three deletion mutants (Δ Ct,

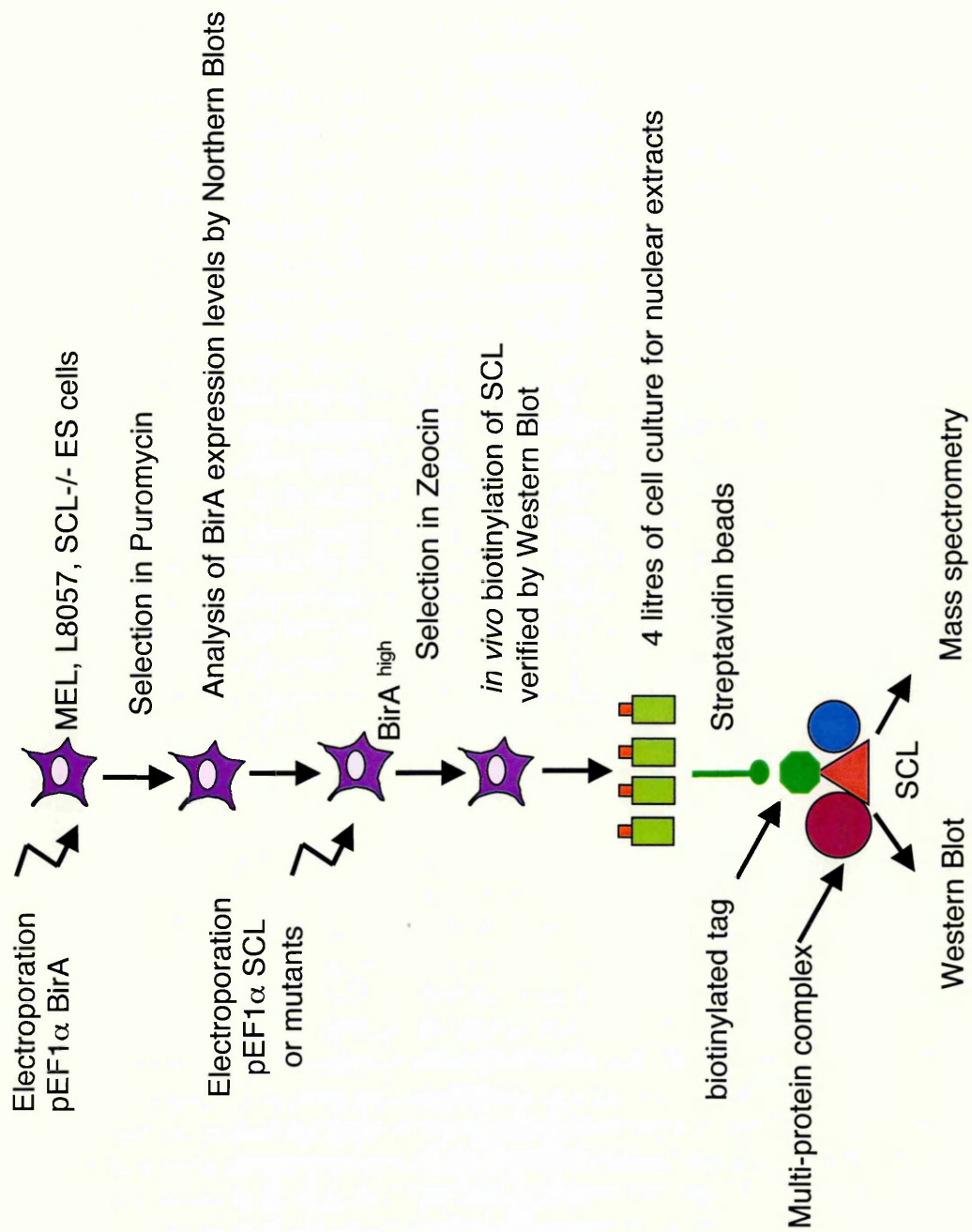


Figure 5.1 Outline of the experimental procedure of the biotin streptavidin purification strategy

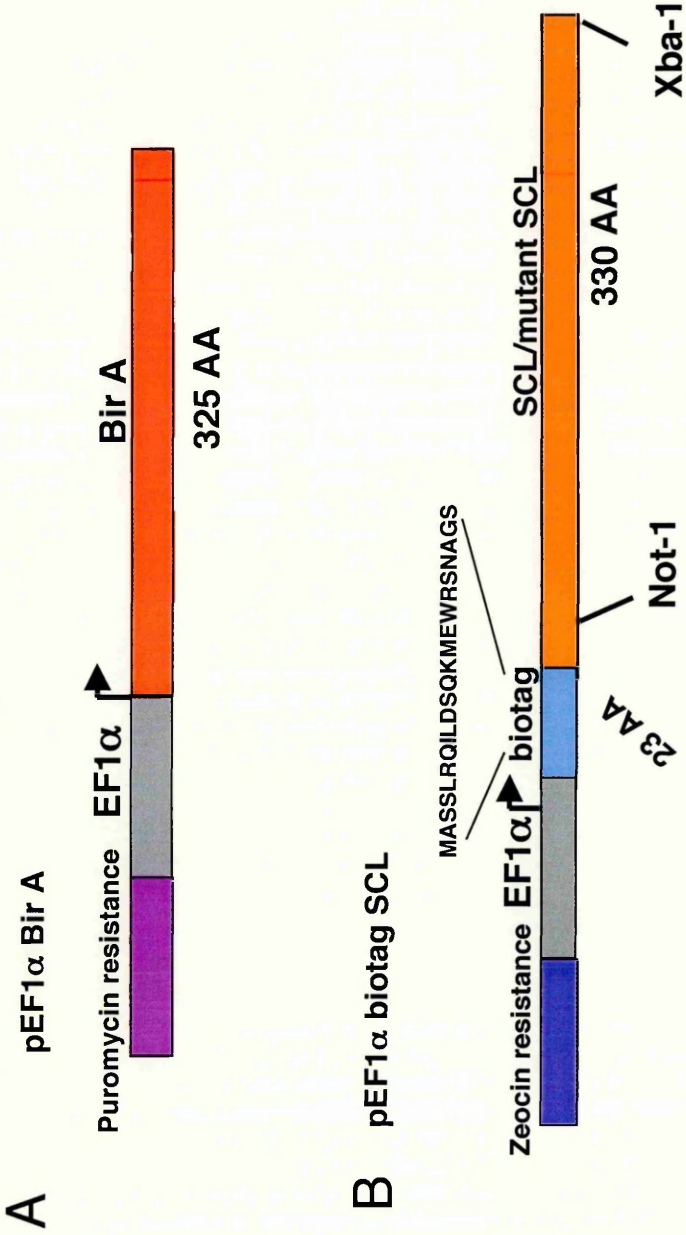


Figure 5.2 The constructs
A: Schematic presentation of the biotin ligase construct
B: Schematic presentation of SCL and SCL mutant constructs

Δ Nt, Δ CtNt), three substitution mutants (RER, FL and H2F-G) and three swapped mutants (SMS, SNS, SES,).

The deletion mutants would help us to broadly map the domains of interaction of SCL with the newly identified partners. The RER mutation abrogates SCL's ability to bind to DNA. The absence of interaction between this mutant and newly identified SCL partners could suggest that direct DNA binding of SCL is required for their interaction. For the FL mutant, two residues in helix 1 crucial for heterodimerisation with E2A were mutated into alanines. Using this mutant in pull-down experiments would show whether heterodimerisation was absolutely required for protein partners to bind to SCL. The H2F-G mutant was defined in the detailed structure-function study of the SCL HLH protein interaction domain¹⁹³. As already mentioned, this study was aimed at identifying some of the crucial aminoacids within the HLH domain of SCL indispensable for primitive and definitive haematopoiesis. A variety of different constructs containing point mutations within the SCL HLH region were assessed for their ability to rescue haematopoiesis from SCL^{-/-} ES cells *in vitro*. In this loss-of-function study, it became apparent that a F-G mutation in helix two (H2F-G) was of particular interest as this mutation did not seem to affect SCL/E2A heterodimerization ability, but abolished rescue of primitive haematopoiesis. We therefore postulated that this phenylalanine in helix two could mediate protein-protein interaction crucial for SCL function and tested the interactions of the H2(F-G) mutant with known partners of SCL, as well as newly identified ones.

Finally, in the swapped mutants, the SCL HLH domain was substituted by the HLH domains of other bHLH protein family members : The muscle-specific class II bHLH protein MyoD, the neuronal class II bHLH protein NSCL and the ubiquitous

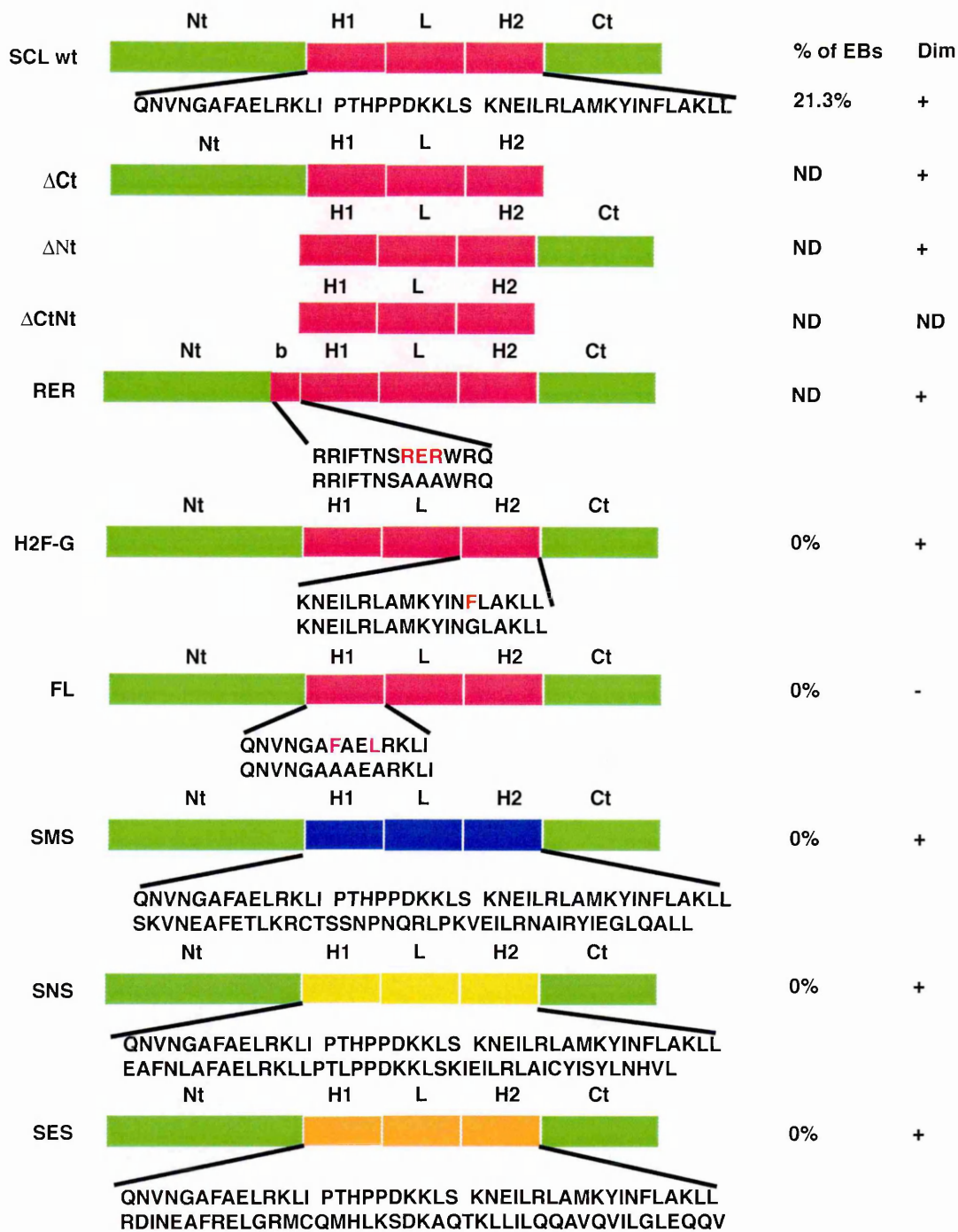


Figure 5.3 Schematic presentation of SCL mutants used in this study
Deletion mutants (ΔCt, ΔNt, ΔCtNt), substitution mutants (RER, H2F-G, FL) and swap mutants (SMS, SNS, SES). The table indicates the percentage of red embryoid bodies (EB) obtained with the respective construct in rescue experiments and the dimerisation ability with E2A (Dim). Ct=C terminal; Nt=N terminal, RER=DNA binding mutant, H2F-G=phenylalanine substitution in helix 2, FL= phenylalanine and lysine in helix 1 replaced by alanines
SMS=SCL HLH domain replaced by that of MyoD
SNS=SCL HLH domain replaced by that of NSCL
SES=SCL HLH domain replaced by that of E47

class I bHLH protein E2A. These swapped mutants would enable us to test the haematopoietic specificity of the protein interactions studied.

All mutated cDNAs were subcloned into pEF1 α biotag vector in replacement of the wild-type SCL sequence (figure 5.2A).

3 RESULTS

3.1 Generation of SCL^{-/-} ES cells, MEL and L8057 cells that co-express the biotin-ligase Bir A and biotagged wild-type or mutant SCL

After transfection of SCL^{-/-} ES cells, MEL and L8057 cells with the pEF1- α Bir A constructs and selection of stable clones, total RNA was extracted from several independent clones and Northern blots were performed using the last 500 nucleotides of Bir A cDNA as a probe (Figure 5.4). Bir A mRNA (1.3kb) was expressed at high levels in all clones compared to wild type controls.

SCL^{-/-} ES cell clone 2, L8057 cell clone 1 and MEL cell clone 8 were subsequently transfected with pEF1- α biotag wild-type (wt) SCL (bio-SCL). L8057 clone 1 and MEL clone 8 were also transfected with the mutant SCL H2(F-G). After selection of stable co-transfected clones, nuclear extracts were prepared and Western Blots performed with α SCL antibody. Endogenous SCL was not detected in MEL and L8057 clones transfected with wt bio-SCL, as only the slow-migrating biotin-tagged protein was observed. In contrast, MEL and L8057 cells transfected with bio-SCL H2(F-G) retained expression of endogenous SCL, as bands corresponding to both tagged and untagged SCL proteins are detected (Figure 5.5, top panel). After stripping the membrane and incubation with a Streptavidin-HRP conjugate,

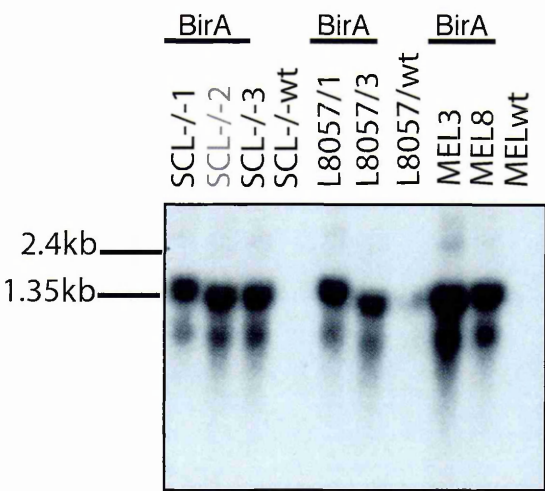


Figure 5.4: The biotin ligase BirA is expressed in transfected clones
Total RNA was extracted from SCL-/- ES cells, L8057 and MEL cell clones transfected with the biotin ligase Bir A as well as from wild-type cell controls. The Northern Blot analysis shows expression of Bir A mRNA (1.3 kb transcript) in all the transfected clones tested.

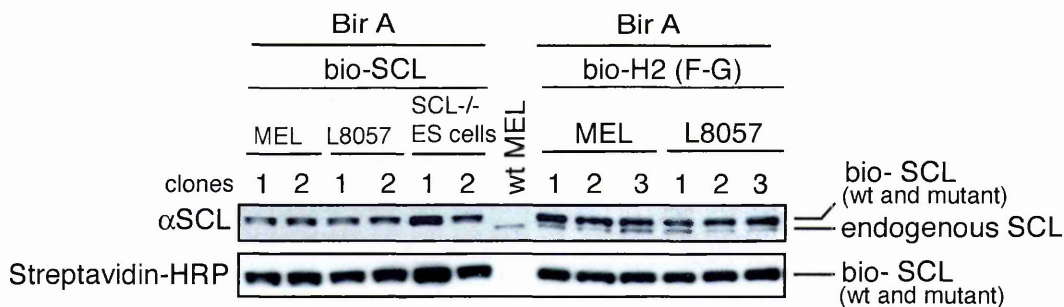


Figure 5.5: SCL is biotinylated in vivo

Nuclear extracts were prepared from non-induced MEL, L8057 and SCL^{-/-} embryonic stem (ES) cells co-expressing biotagged SCL or the H2 (F-G) mutant and the BirA biotin ligase and from wild-type MEL cells. Western Blots were performed using anti-SCL antibodies (top panel; note that expression of biotagged SCL suppresses expression of endogenous SCL in MEL and L8057 cells). The membrane was then stripped and incubated with streptavidin-HRP to confirm that biotagged SCL was biotinylated *in vivo* (bottom panel). bio-SCL=biotinylated SCL, HRP= horse raddish peroxidase

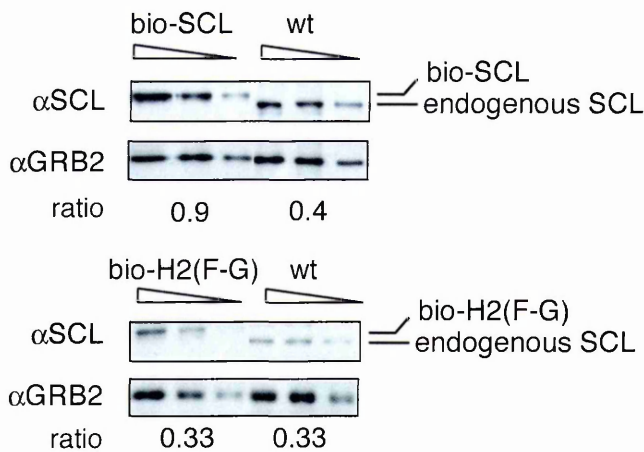


Figure 5.6: Quantification of SCL protein expression in transfected MEL cells

Relative expression levels of SCL in nuclear extracts prepared from wild-type MEL cells and from clones expressing bio-SCL (wild-type or H2(F-G) mutant) were analysed by Western Blot. The protein GRB2 served as an internal loading control. Triangles represent serial dilutions of the input. Immunoblots were quantified using the Quantity One Software from Biorad and ratios between levels of expression of SCL and GRB2 calculated.

presence of biotinylated SCL was confirmed in all the clones (figure 5.5, bottom panel).

Down-regulation of expression of the endogenous SCL gene in MEL and L8057 clones transfected with wt bio-SCL (and possible perturbation in clones expressing the bio-SCL variant), precluded any direct comparison of levels of expression of the transgenes versus the endogenous gene. Therefore, to select MEL and L8057 clones with levels of bio-SCL comparable to that of endogenous SCL, levels of expression of bio-SCL and of a loading control (GRB2) in transfected cells, and of wt SCL and GRB2 in untransfected cells were compared and ratios calculated. Transfected clones that showed ratios comparable to that of untransfected cells were retained for further analyses (figure 5.6 and data not shown).

3.2 Primitive and definitive haematopoiesis from SCL^{-/-} ES cells is restored upon expression of biotinylated SCL in an *in vitro* rescue assay

Before characterising SCL-containing complexes in MEL and L8057 cells, we checked that the presence of a biotinylated tag in the N-terminus of SCL did not perturb the function of the protein. To do this, we took advantage of the rescue assay of SCL-null ES cells whereby introduction of a wt SCL cDNA into these cells fully restores haematopoietic development⁸⁹. A general description of this two step rescue assay^{89,261} is given in figure 5.7: Upon primary replating, ES cells give rise to three-dimensional cellular structures called embryoid bodies (EB) containing, amongst others, haematopoietic precursors. In contrast to wild-type ES cells, SCL^{-/-} ES cells form white embryoid bodies devoid of primitive erythroid cells after primary replating and develop no haematopoietic activity after secondary replating. However, when SCL null cells are transfected with wild-type SCL cDNA, both primitive and definitive haematopoietic activities are restored. We have used

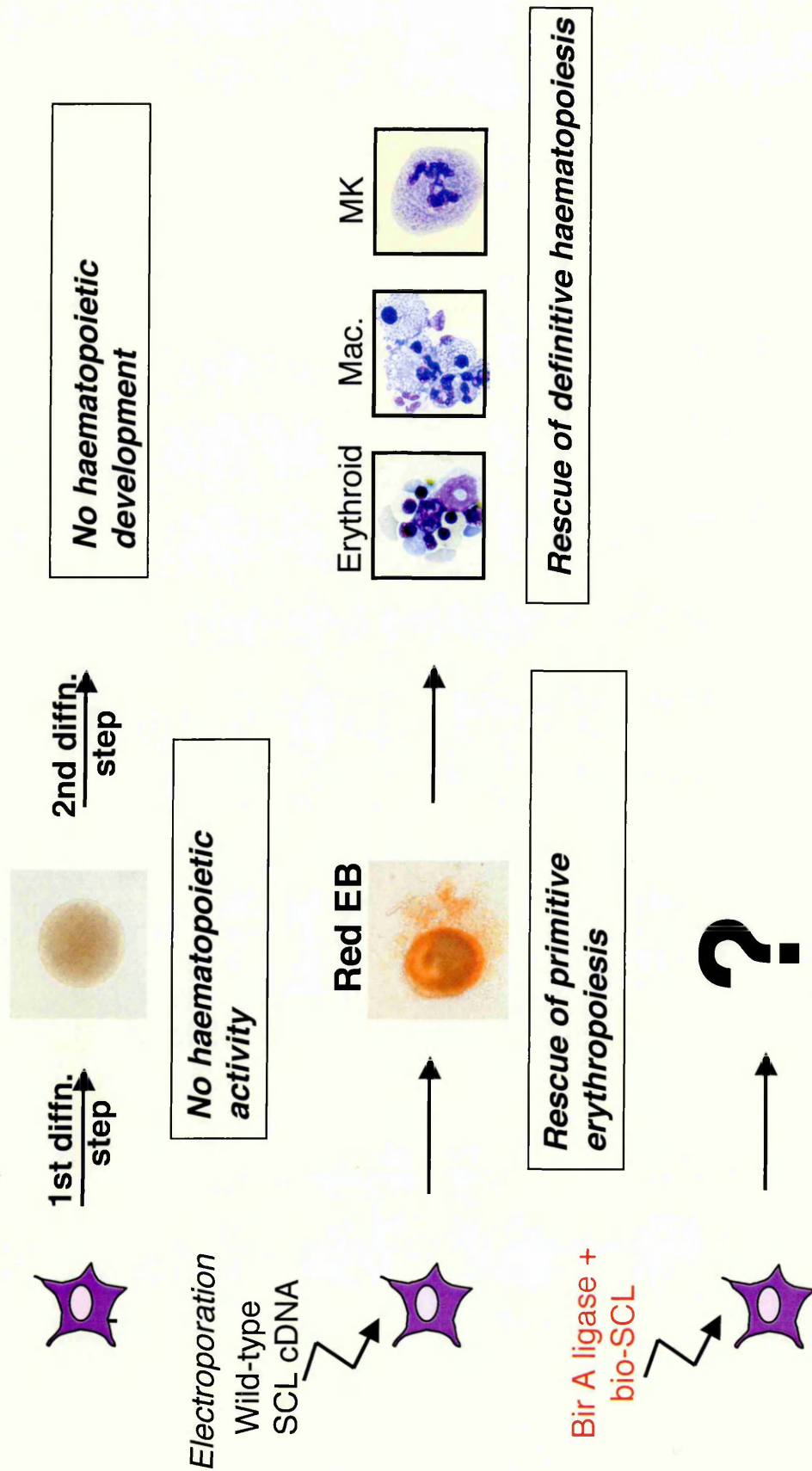


Fig 5.7 *In vitro* haematopoietic differentiation assay

Using different cytokine cocktails, wt ES cells can differentiate in a two step *in vitro* assay into all haematopoietic lineages. SCL^{-/-} ES cells do not give rise to primitive and definitive haematopoiesis. However, haematopoietic development can be fully rescued upon reintroduction of wt SCL cDNA into these cells (Porcher1996). We have used this system to test whether biotinylated SCL used in this study was functional.

MAC=macrophages; MK=megakaryocytes; EB=embryoid bodies

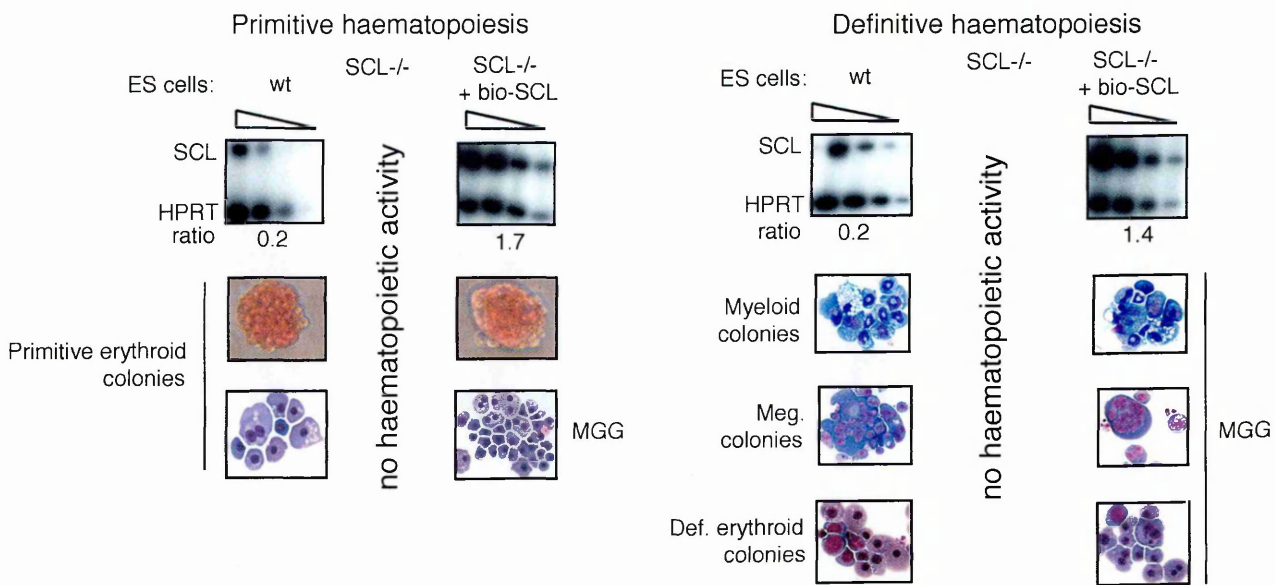


Figure 5.8: Biotinylated SCL is able to rescue primitive and definitive haematopoiesis from SCL-/- ES cells in an in vitro differentiation assay

SCL-/- ES cells expressing BirA biotin ligase and biotagged SCL were differentiated into primitive and definitive haematopoietic lineages. Wt ES cells and SCL-/- cells served as controls. Levels of SCL expression were analysed in primitive erythroid colonies (left top panels) and definitive erythroid colonies (right top panels) by semiquantitative RT-PCR and relative to levels of expression of the HPRT gene. Morphology was assessed by phase contrast microscopy (left panel, primitive erythroid colonies) and May-Grunwald-Giemsa staining (MGG panels). Meg=megakaryocyte def=definitive wt=wild-type

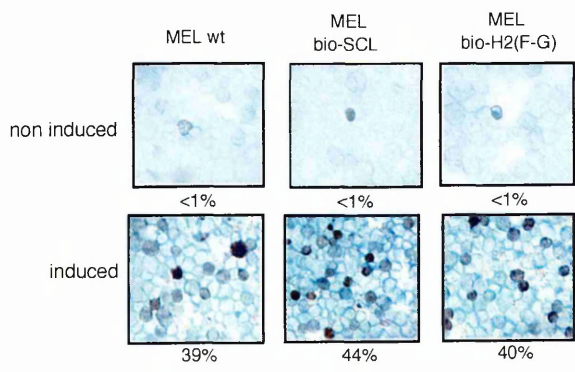


Figure 5.9 Biotinylated SCL does not promote spontaneous differentiation of MEL cells
Benzidine staining of non-induced and induced wt MEL cells and MEL cells expressing biotinylated SCL or the H2 (G-F) mutant. Percentages refer to the number of Benzidine positive cells counted.

this system to assess functionality of our construct. To this, SCL^{-/-} ES cells stably transfected with the biotin ligase and with pEF-1 α bio-SCL were differentiated into primitive and definitive haematopoietic cells. Despite high levels of bio-SCL transcripts as judged by semi-quantitative RT-PCR of RNA isolated from haematopoietic colonies (7 to 8.5-fold higher than endogenous SCL, figure 5.8, top panels), complete rescue of primitive and definitive hematopoiesis was observed. Primitive erythroid colonies (figure 5.8, left panel) and definitive erythroid, megakaryocytic and myeloid colonies (data not shown) derived from cells expressing biotinylated SCL looked morphologically identical to their wt counterparts. May-Grunwald-Giemsa staining showed terminal cellular maturation of cells derived from rescued ES cells similar to that derived from wt cells (figure 5.8, MGG panels). Moreover, the number of haematopoietic progenitors derived from rescued ES cells was similar to that derived from wt ES cells, as observed upon colony assay (data not shown). We concluded that biotinylation of SCL did not seem to perturb any function critical for haematopoietic specification and maturation of haematopoietic lineages.

3.3 Biotinylated SCL protein does not induce spontaneous differentiation of MEL cells and allows normal induction by HMBA or DMSO

Previous studies reported that over-expression of SCL in MEL cells induced spontaneous terminal maturation²¹⁰. To check whether this applied to the MEL clones expressing bio-SCL or bio-H2(F-G), benzidine staining was performed before and after exposure of the cells to an inducer of terminal differentiation. Cells non-exposed to the inducing agent did not show any enhanced number of benzidine-positive cells as compared to wt MEL cells (figure 5.9, top panel). A proportion of these cells was however able to undergo terminal maturation as

shown by the substantial number of benzidine-positive cells after treatment with the inducing agent (figure 5.9, bottom panel).

In conclusion, we show that the function of SCL in haematopoietic cells is not affected by biotinylation. Moreover, we generated MEL and L8057 clones expressing biotinylated SCL (wt and mutant) at levels similar to that of the endogenous protein in wt cells, thereby preserving a normal protein stoichiometry.

3.4 The known pentameric SCL complex is present in pull downs from MEL and L8057 cell nuclear extracts

Cells contain endogenously biotinylated proteins or peptide sequences that might be recognised by the biotin ligase BirA. Therefore, to be able to distinguish between proteins specifically pulled-down upon SCL biotinylation and background proteins, crude nuclear extracts were prepared from MEL and L8057 clones co-expressing bio-SCL and BirA ligase as well as expressing BirA alone. Purification of SCL-containing multiprotein complexes was achieved upon pull-downs with streptavidin beads, under low stringency conditions (150mM NaCl, 0.3% Nonidet P40). After washes in more stringent conditions (250mM NaCl, 0.3% Nonidet P40), complexes were eluted and analysed by Western Blot. First, presence of bio-SCL and bio-H2(F-G) in the fraction precipitated from MEL and L8057 nuclear extracts was confirmed; as expected, no SCL was purified from cells expressing BirA only (figure 5.10, PD fractions). Most of biotinylated SCL bound to streptavidin beads as there was very little to no product in the unbound (UN) fractions. We next checked whether known partners had co-purified with wt bio-SCL. We were able to detect the presence of E2A, HEB, LDB-1 and LMO2 in PD fractions using 250 mM NaCl washes, thereby validating the strategy. Importantly, none of these proteins were detected in the PD fraction from cells transfected with BirA only, confirming that

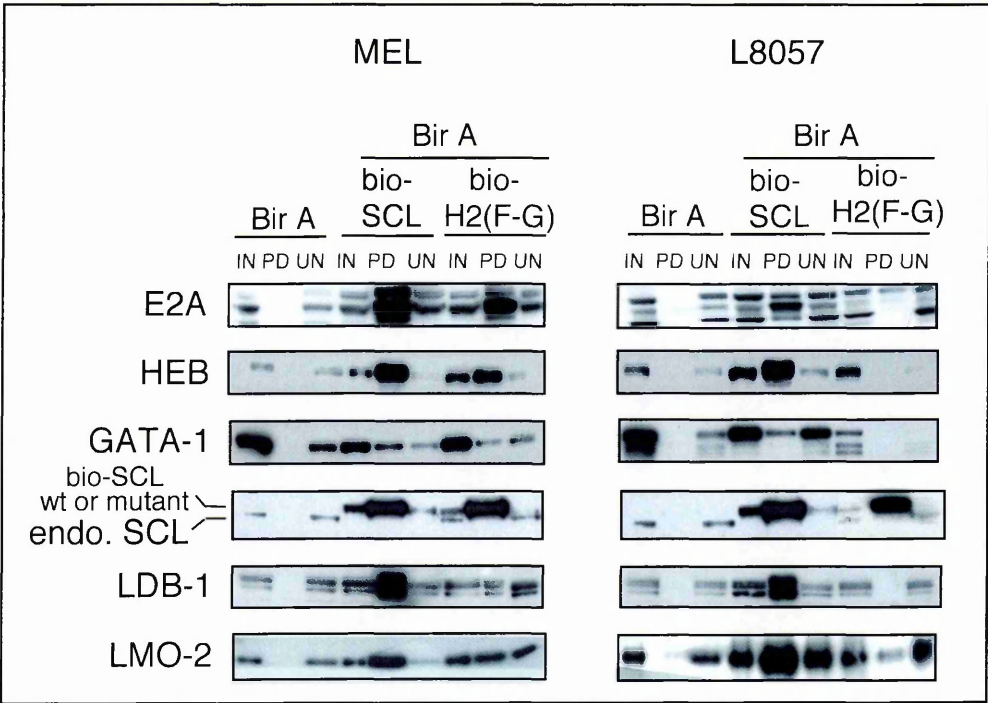
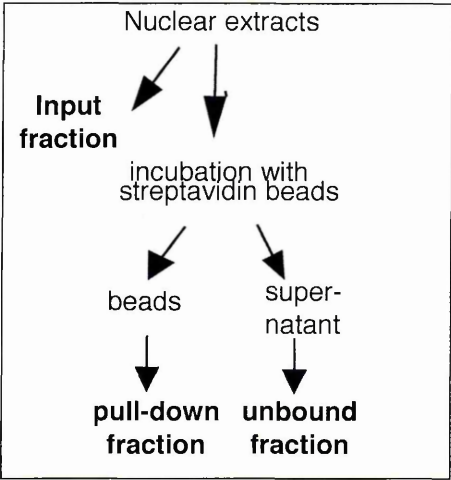


Figure 5.10 Known partners of SCL co-purify with bio-SCL

Pull-downs of SCL-containing complexes (strategy depicted at the top) were performed using nuclear extracts of MEL (bottom panel left) and L8057 (bottom panel right) cells transfected with biotin ligase BirA only (as a negative control) or with Bir A and bio-SCL or bio-H2(F-G).

Eluates were subjected to Western Blot analysis with antibodies against known partners of SCL (E2A, HEB, Ldb-1, LMO2, GATA-1).

IN: input; PD: pull-down; UN: unbound.

they had been specifically pulled-down upon biotinylation of SCL. Interestingly, the interaction with GATA-1 could only be detected after reducing the stringency of the washes to 150 mM NaCl. Pull-down profiles observed from MEL and L8057 cells were similar with substantial enrichment for E2A(E12/47), HEB, LMO2 and LDB-1. Enrichment of GATA-1 was not as drastic, suggesting that only a fraction of GATA-1 might be involved in SCL-containing protein complexes or reflecting the weak nature of the interaction between the two proteins.

3.5 Phenylalanine in SCL helix 2 is critical for LMO-2 and LDB-1 binding

The point mutation in helix 2 of the SCL HLH region leads to a complete absence of primitive haematopoiesis in the *in vitro* haematopoietic rescue assay from SCL-null ES cells, despite the fact that heterodimerization with E12/E47 is not impaired¹⁹³. We therefore hypothesised that this mutant would no longer be able to bind to some of the critical partners of SCL. As shown in figure 5.10, LMO-2, LDB-1 and GATA-1 binding is significantly reduced in MEL cells. Interestingly, the H2F-G binding defect of LMO-2, LDB-1 and GATA-1 was even more pronounced in L8057 clones, very likely due to the impairment of heterodimerization with E12/47 and HEB (figure 5.10, right panel, PD fractions).

3.6 Identification of potential candidate protein partners of SCL

To identify additional putative partners, SCL-containing complexes were then purified from 5 mg of crude nuclear extracts from MEL and L8057 cells co-expressing BirA and bio-SCL under low stringency conditions (150mM NaCl, 0.3% Nonidet P40) and the nature of the precipitated products analysed by mass spectrometry. In order to be able to distinguish between proteins specifically pulled-down upon SCL biotinylation and background binding proteins, cells expressing BirA only were analysed in parallel. First, upon separation of the pull-down

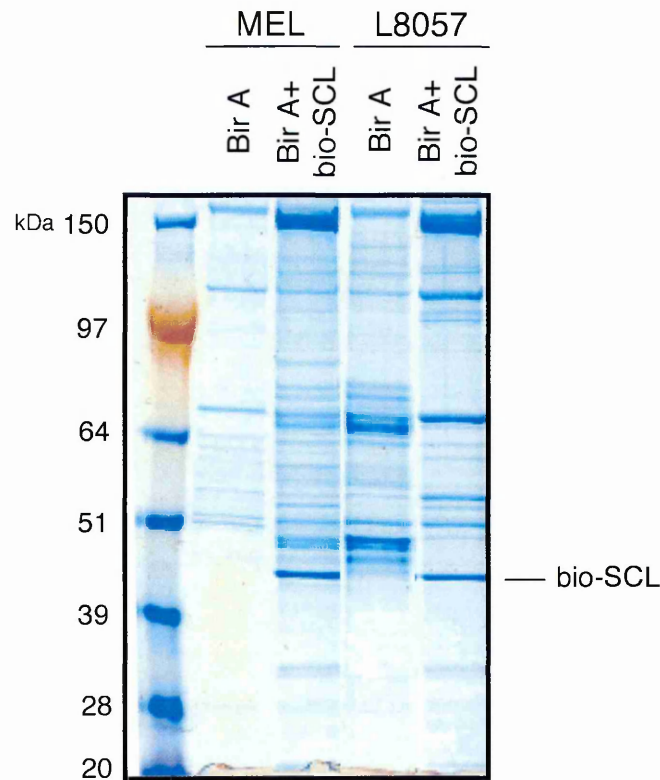


Figure 5.11 Coomassie stained gel of representative pull-down experiments

Nuclear extracts prepared from MEL and L8057 clones transfected with BirA only or co-transfected with biotagged SCL and BirA were subjected to pull-downs with streptavidin beads. Complexes were eluted, separated by SDS-Page and stained with Coomassie blue.

Bands observed in the fraction pulled-down from cells expressing BirA only correspond to endogenously biotinylated proteins or peptide sequences recognized by BirA biotin ligase. In the fractions pulled-down from cells expressing bio-SCL, there is enrichment in bands that do not appear in the BirA-only controls.

All lanes, including the negative controls, were cut into gel slices, digested with trypsin and subject to liquid chromatography mass spectrometry (LC-MS – service provided by Functional Genomics and Proteomics Laboratories at the University of Birmingham).

products on SDS-PAGE, Coomassie-blue staining revealed different patterns between the control cells and cells expressing bio-SCL (figure 5.11). From MEL cells expressing bio-SCL, there was a significant enrichment in bands that did not appear in the BirA-only control. From L8057 cells, although the control lane showed more background proteins, there were also significant differences with cells expressing bio-SCL. The background proteins identified by mass spectrometry were similar to those reported previously²⁶². They consisted mainly in naturally biotinylated proteins such as carboxylases and their co-enzymes, as well as splicing factors, ribosomal proteins and proteins associated with the cytoskeleton such as actin and tubulin (table 5.1). By contrast, upon SCL biotinylation, we detected some of the known partners of SCL, as well as additional transcription factors, co-factors, chromatin remodelling proteins that were absent from the control BirA-only experiments (table 5.2).

SCL was the most abundant protein detected, followed by its heterodimerisation partners, the E-proteins (E12/E47, E2.2). Another member of the pentameric complex, LDB-1, was found in both MEL and L8057 cells. However, we never identified GATA-1 or LMO-2 by mass spectrometry (see discussion).

In addition to these known partners, a significant number of new potential candidates was identified (table 5.2). In an attempt to validate these results, we checked the interaction between SCL and selected newly identified proteins by Western Blot analysis from PD fractions (data not shown). Most of the proteins we tested were also weakly present in the PD fractions from BirA-only cells. It is possible that more stringent binding conditions are necessary to abolish non-specific binding to streptavidin beads. Some of the protein interactions were not always reproducible even in low stringency washes. This may reflect the weakness

TABLE 5.1

background binding proteins in MEL and L8057 cells

| Protein ID | biological process | remarks |
|---------------------|--------------------|-----------------------------|
| Carboxylases | metabolism | require biotin as co-enzyme |
| acetyl transferases | metabolism | require biotin as co-enzyme |
| actin | cytoskeleton | |
| myosin | cytoskeleton | |
| tubulin | cytoskeleton | |
| ribosomal proteins | protein synthesis | |
| splicing factors | RNA processing | |

Table 5.1 Background binding proteins

These proteins were identified by mass spectrometry in pull-downs performed from nuclear extracts of clones transfected with BirA only.

TABLE 5.2

| MEL | | | |
|-------------------------|----------------|----------------------------|--------------------------------|
| Protein ID | No of peptides | biological process | remarks |
| E12/E47 | 10 | transcription factor | known partner |
| E2.2 | 9 | transcription factor | known partner |
| LDB-1 | 5 | transcription factor | known partner |
| SCL | 11 | transcription factor | |
| SSDP | 5 | transcription factor | interacts with Ldb-1 |
| ETO-2=MTG16 | 6 | co-repressor | |
| TIF1 γ + β | 6 | chromatin remodelling | |
| LAP2=thymopoietin | 7 | nuclear architecture | interacts with Rb |
| FACT 40 | 4 | basal transcription | nucleosome shifting |
| SSRP-1 | 3 | basal transcription | nucleosome shifting |
| RbAp 46 | 3 | co-repressor | interacts with Rb |
| HDAC class 1 | 4 | chromatin remodelling | co-repressor |
| RUV B like1 (TIP49) | 2 | chromatin remodelling | co-activator |
| DEAD (p68) | 7 | ATP dependent RNA helicase | interacts with CBP and p300 |
| HSP 70 | 17 | chaperone | |
| EWS | 4 | transcription co-factor | interacts with basal machinery |
| KU 70/80 | 6 | DNA repair | |
| TCP(TRiC) | 2 | chaperone | HDAC-3 priming |

| L8057 | | | |
|------------|----------------|----------------------------|-----------------------------|
| Protein ID | No of peptides | biological process | remarks |
| E12/E47 | 17 | transcription factor | known partner |
| HEB | 2 | transcription factor | known partner |
| LDB-1 | 8 | transcription factor | known partner |
| SCL | 21 | transcription factor | |
| SSDP | 2 | transcription factor | interacts with Ldb-1 |
| ETO-2 | 5 | co-repressor | |
| KAISO | 2 | co-repressor | |
| LCP-1 | 2 | unknown | |
| DEAD (p68) | 3 | ATP dependent RNA helicase | interacts with CBP and p300 |
| HSP 70 | 16 | chaperone | |

Table 5.2 SCL candidate partners

These proteins were identified by mass spectrometry as potential candidate partners of SCL. They were not identified in the pull-downs performed from clones transfected with BirA only.

of binding to SCL. Therefore, further analysis will be required to confirm the reality of most of these interactions.

However, two proteins, single-stranded DNA binding protein 2 (SSDP2) and Eight-Twenty-One 2 (ETO-2), consistently came up in mass spectrometric analysis with multiple hits, and were chosen for further analysis.

4 DISCUSSION

Strengths and weaknesses of the technique

The biotin-streptavidin approach allowed us to successfully and robustly pull-down SCL containing multiprotein complexes. In comparison to the conventional immunoaffinity purification technique this approach had several major advantages:

- 1) Due to the high affinity between biotin and streptavidin, it permits purification of protein complexes from crude nuclear extracts in a single step, and
- 2) achieves protein yields high enough for easy analysis by mass spectrometry.
- 3) It allowed us to analyse the protein-binding characteristics of SCL mutants.

However, a major downside of this approach could be the perturbation of the composition of SCL-containing complexes by expression of exogenous, biotinylated protein. It is therefore critical to set up the system carefully: Importantly, we first showed by rescue experiments from SCL^{-/-} ES cells that the function of SCL in haematopoietic cells is not affected by biotinylation. We then generated MEL and L8057 clones expressing biotinylated SCL (wt and mutant) at levels similar to that of the endogenous protein in wt cells, thereby preserving a normal protein stoichiometry. It was therefore likely that the protein-protein

interactions involving bio-SCL would reflect the interactions observed with endogenous SCL.

The composition of SCL-containing complexes may be different in MEL and L8057 cells

We first validated the pull-down strategy by showing that the SCL pentameric complex can be isolated from MEL and L8057 cells upon biotinylation of wild-type SCL. Moreover, this technique helped us test one of the SCL mutants characterised in a recent structure-function study¹⁹³ for its ability to bind to SCL protein partners in MEL and L8057 cells. Thus, we identified the phenylalanine in helix 2 of SCL as critical for binding to LMO-2 in MEL cells, whereas heterodimerisation with E2A was not affected. It has been described previously, that LMO-2 physically interacts with SCL, whereas LDB-1 links LMO-2 to GATA-1²¹⁹. It is therefore not surprising that we also saw a reduction in LDB-1 and GATA-1 binding in the H2 mutant cells.

In L8057 cells, however, this mutant led to almost complete absence of all protein interactions studied, thereby indicating that, although the same core complex (comprising SCL, E2A or HEB, LMO2, LDB1 and GATA-1) is present in the two cell types, there may be differences in the overall composition of the SCL-containing complexes or in the strength of the interactions between proteins. Mutation of a critical residue in SCL could therefore destabilise the complexes differently.

Furthermore, the results from the mass spectrometry analysis of streptavidin pull-downs show that, in MEL cells, SCL is a partner of multiple other proteins, whereas in L8057 cells only few candidates were identified. If these results are validated, this further supports the idea that SCL-containing complexes are indeed different in these cell types.

Discussion of mass spectrometry results

Known partners

Upon mass spectrometric analysis of the pull-down products we were able to identify known partners of the pentameric complex, thus validating the technique. However, neither GATA-1 nor LMO2 were identified. For GATA-1, this is perhaps not surprising as Western Blot analysis of material isolated from cells expressing biotinylated SCL showed that the SCL/GATA-1 interaction was salt-sensitive and only a fraction of GATA-1 interacts with SCL, thus making its detection by mass spectrometry difficult. LMO2 is a small protein (20 kDa) and, therefore, the peptides resulting from trypsin digestion are likely to be under-represented in the mass spectrometry analysis as opposed to larger proteins.

The majority of interactions found involve repressor complexes

In addition to these known partners, the biotin-streptavidin approach allowed us to identify a number of novel interesting candidate partners of SCL, although the interaction between the majority of them and SCL remains to be validated.

Amongst them is the co-activator and ATP-dependent RNA helicase p68 known to interact with CBP/p300³⁰³. Interestingly, CBP/p300 was shown to be a protein partner of SCL¹⁹⁸.

Perhaps surprisingly, the majority of the proteins identified are part of repressor complexes. Interestingly, two of them, Lamin A/C binding protein LAP2 and the retinoblastoma associated protein 46 (RbAp46) have been shown to interact with retinoblastoma protein³⁰⁴ and are implicated in gene repression. As mentioned in chapter IV, SCL was shown to repress *c-kit* gene expression by recruiting retinoblastoma protein²²⁶, and our results from the immunopurification experiments suggest that SCL might also interact with p107.

Besides, several of these putative partners involved in transcriptional repression have also been identified by quantitative mass spectrometry using isotope-coded affinity tag (ICAT) as partners of NF-E2p18/MafK during erythroid differentiation (TIF γ , RbAp46, HDAC1)¹¹⁷. This might indicate that, in erythroid cells, these proteins are part of general multi-protein complexes that bind to sequence-specific transcription factors such as NF-E2 or SCL.

The reasons why the majority of putative partners we identified are involved in repression of gene transcription remain speculative. However, we have to consider that the purification strategy using salt extraction for nuclear extract preparation is biased towards the isolation of repressor complexes. This is in accordance to the results obtained from the purification of GATA-1 complexes in MEL cells by the same technique (John Strouboulis, unpublished data). Besides, it might be that repressor complexes are more abundant in the nucleoplasm or that they come off DNA more easily than activating complexes.

In conclusion, we show that the biotin-streptavidin purification technique can be used successfully to pull-down SCL-containing multiprotein complexes from two different haematopoietic cell lines. As presented in the next chapter, this has opened up a new area of investigation regarding SCL function in erythrocytes and megakaryocytes.

CHAPTER VI: CHARACTERISATION OF PREVIOUSLY UNIDENTIFIED SCL PARTNERS

1 INTRODUCTION

After successfully setting up and validating the biotin-streptavidin purification strategy, I further analysed the results obtained by mass spectrometry.

Possible SCL protein partners identified by MS with more than one peptide hit and not present in MS results from biotin ligase only clones were chosen for validation experiments comprising several different steps.

As already mentioned in chapter V, we tested the interaction between newly identified candidate protein partners in biotin pull-downs by Western Blot. Western blotting is sensitive up to the femtomole detection level, whereas MS requires picomoles of protein. This approach therefore helped us to exclude false positive candidate partners that had not appeared in the MS results of Bir A only pull-downs and to verify the mass spectrometry results.

We then chose two proteins, SSDP2 and ETO-2 for further analysis.

1.1 SSDP2

SSDP2 was originally cloned from a chick embryo fibroblast cDNA expression library³⁰⁵. It specifically binds to a pyrimidine-rich DNA region within the $\alpha 2$ *collagen* gene. This region is unusual in that it forms single strands and is hypersensitive only when the promoter is active thus implying that it may be important for transcriptional regulation of the *collagen* gene. SSDP2 is highly conserved throughout all species. Importantly, a complex comprising SSDP2,

LDB1/Chip and the LIM-homeodomain protein Apterous was described in the wing in *Drosophila* ²⁶³, In *Xenopus*, SSDP enhances axis induction by LDB-1 in conjunction with the LMO-2 ortholog Xlim-1³⁰⁶. Together, these studies reveal SSDP as a co-factor of LDB-1 in invertebrate and vertebrate development. However, nothing is known so far about its role in adult haematopoiesis.

1.2 ETO-2

ETO-2 is a member of the ETO family of co-repressor proteins whose founding member (ETO, MTG8) was originally identified upon characterisation of the t(8;21) chromosomal breakpoint, involved in 12-15% of acute myeloid leukaemia (AML) (for review, ³⁰⁷). In mouse, ETO-2 is expressed during embryogenesis, in most adult tissues and in myeloid and erythroid cell lines ²⁶⁴. MTG16, the human homolog of ETO-2, was identified by molecular characterisation of the t(16;21) associated with therapy-related AML. It is highly expressed in various haematopoietic organs such as spleen, thymus and peripheral blood leukocytes ³⁰⁸. Gene targeting experiments of ETO with whom it shares a 75% homology, have shown its vital role in the development of the gastrointestinal system. Apart from this, most functional data on ETO and ETO-2 stems from the assignment of their interacting protein partners³⁰⁹. The first direct evidence supporting the co-repressor model came from the isolation of the human nuclear receptor co-repressor NCoR in a yeast two-hybrid assay using full length ETO as bait^{130,131}. Subsequently, other partners were identified such as SMRT¹²⁸ and mSin3A¹³¹, all of which interact with HDACs.

2 OUTLINE OF EXPERIMENTS

To further validate the interaction between SSDP2 and SCL, as there are no α SSDP2 antibodies available, we subcloned SSDP2 cDNA into the pEF1 α -biotag vector and expressed biotinylated SSDP2 in MEL cells. This allowed us to perform pull-downs using the biotinylated protein and subsequent Western Blot with α SCL antibodies. We are now planning to generate antibodies against SSDP2. We then focused on the characterisation of the SCL/ETO-2 interaction. To do this, we have set up a series of experiments that are described below:

- 1) We confirmed the interaction between SCL and ETO-2 by reverse co-immunoprecipitation from untransfected cell lines to document interactions between the endogenous proteins.
 - 2) To exclude the possibility that ETO-2 was pulled-down non-specifically because of contamination with DNA in the input fraction, we repeated the co-IP on DNAase-treated nuclear extracts.
 - 3) We validated the interactions in co-localisation experiments in MEL and L8057 nuclei.
 - 4) Next, we documented the ETO-2/SCL interaction in mouse primary erythroid cells and megakaryocytes.
 - 5) We then mapped the domain of SCL necessary for the interaction with ETO-2 and tested chosen SCL mutants (see Chapter V), for their ability to bind ETO-2.
 - 6) In order to further characterise the nature of the complexes containing SCL and ETO-2, we performed immunodepletion experiments and further co-immunoprecipitations from MEL and L8057 nuclear extracts.
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- 7) As a first step towards the functional characterisation of the new interaction, we used transactivation assays in a heterologous cell line to assess the effect of ETO-2 on reporter gene activation by SCL and the pentameric complex.
 - 8) Finally, we examined changes in the composition of SCL complexes during erythroid differentiation using an *in vitro* differentiation assay of day 12.5 fetal liver cells.

3 RESULTS

3.1 Validation of interesting candidate partners

In a first attempt to validate the mass spectrometry results, we confirmed the interaction between SCL and SSDP2 or ETO-2 by Western Blot analysis from the pulled-down fractions used for MS analysis (data not shown).

3.1.1 SSDP2 is a partner of SCL in erythroid cells

To further validate these results, we then expressed biotinylated SSDP2 in MEL cells and isolated co-purifying proteins with streptavidin beads. We show that SSDP2 interacts with SCL and its partners (the E2A proteins, Ldb1 and LMO2) by Western Blot (figure 6.1).

3.1.2 SCL interacts with ETO-2 in erythroid cells and megakaryocytes

Reverse co-immunoprecipitation

We next validated the interaction between endogenous SCL and ETO-2 by co-immunoprecipitation (coIP) of ETO-2 with SCL antibodies (data not shown) and reverse coIP from MEL cell nuclear extracts (figure 6.2 A). Increasing amounts of ETO-2 antibodies precipitate increasing amounts of SCL, thereby showing the specificity of the interaction. We also confirmed the interaction in L8057 cells

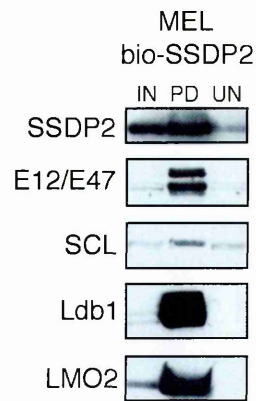


Figure 6.1: The SCL/SSDP2 interaction is confirmed using biotinylated SSDP2

Nuclear extracts were prepared from MEL cells transfected with biotin ligase and bio-SSDP2. Western Blot analysis of streptavidin pull-downs showed that SSDP2 interacts with SCL and its known partners E12/47, Ldb1 and LMO2.

IN=input of crude nuclear extract, PD= streptavidin pull-down, UN=unbound

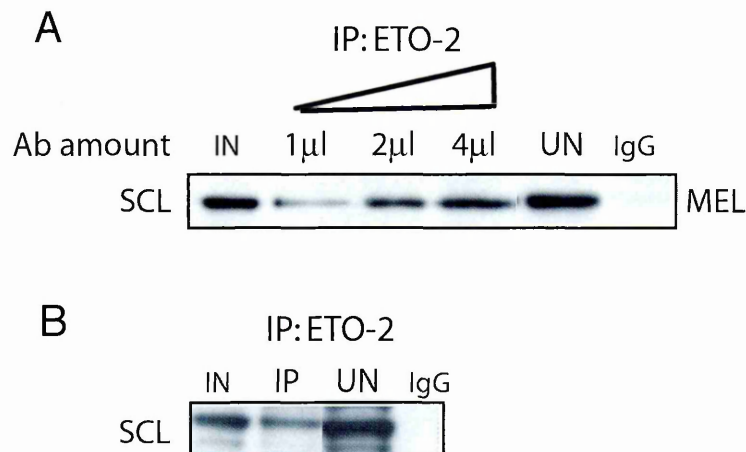


Figure 6.2: The SCL/ETO-2 interaction is confirmed by reverse co-immunoprecipitation of endogenous proteins

A Immunoprecipitation (IP) of SCL from wild-type MEL nuclear extracts with α -ETO-2 antibodies confirms the interaction between the two proteins in an erythroid cell line. Increasing amounts of α -ETO-2 antibody represented by the triangle, precipitate increasing amounts of SCL, thus indicating the specificity of the binding.

B Immunoprecipitation (IP) of SCL from wild-type L8057 nuclear extracts with α -ETO-2 antibodies confirms the interaction between the two proteins in a megakaryoblastic cell line. Ab=antibody, IN=input of crude nuclear extract, UN=unbound, IgG=negative control

(figure 6.2 B). Then, we show by reverse coIP that the SCL/ETO-2 interaction is conserved in induced MEL cells (figure 6.3). Finally, to exclude the possibility that ETO-2 could have been purified because of non-specific binding to nucleic acids, nuclear extracts were treated with DNase before coIP. SCL was still detected in the immunoprecipitated fraction, confirming the interaction of ETO-2 and SCL (figure 6.3).

Gel filtration

Upon gel filtration of MEL nuclear extracts, Western Blot analysis of gel filtration fractions shows that ETO-2 elutes in the same high molecular weight fractions as SCL (figure 6.4). MTG16, the human homolog of ETO-2, was found to interact with the zinc finger oncoproteins Gfi-1 and Gfi-1b in *in vitro* assays³¹⁰. As this latter is crucial for erythropoiesis and megakaryopoiesis³¹¹, we reasoned that it could interact with ETO-2 in mouse haematopoietic cells and, as a first indication of this, checked its fractionation profile. We found that Gfi-1b elutes in the same high molecular weight fractions as ETO-2 and SCL.

Co-localisation

Finally, to further document the interaction between SCL and ETO-2, we have performed co-localisation experiments. These experiments were kindly performed by Dr Francisco Iborra (MHU). MEL and L8057 cells were fixed and immunolabelled with anti-SCL and anti-ETO-2 antibodies. For both proteins and in both cell types, the staining appeared nuclear when compared with DAPI staining (data not shown) and punctate, as previously reported in other cell types^{156,264} (figure 6.5, top panel and data not shown). In a first attempt to demonstrate co-localisation of the proteins, we have undertaken a conventional analysis. Dual staining of the cells showed that a substantial amount of SCL and ETO-2 co-

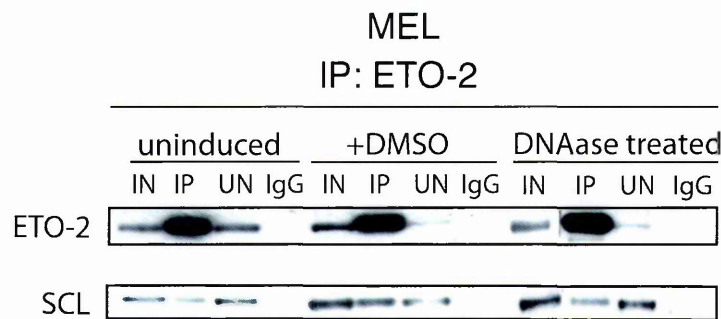


Figure 6.3 The SCL/ETO-2 interaction is present in induced MEL cells and in DNAase treated nuclear extracts

Nuclear extracts were prepared from uninduced and DMSO-induced MEL cells. A fraction of the nuclear extract from uninduced cells was treated with DNAase. All nuclear extracts were subjected to co-immunoprecipitation using anti-ETO-2 antibody. Western Blots were probed with α ETO-2 antibody (upper panel) and α SCL (lower panel) antibodies.

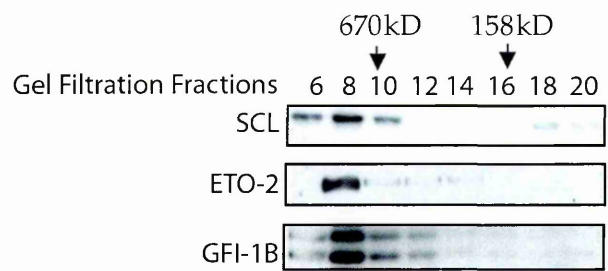


Figure 6.4 Gelfiltration of wild-type MEL nuclear extracts

Nuclear extracts prepared from wild-type MEL cells were fractionated on a Superose 6 H/R column. Western blots of fractions 6 to 20 were probed with the antibodies as indicated. This revealed that SCL co-migrates with ETO-2 and GFI-1B in the high molecular weight fractions. Molecular weights in kD are indicated at the top.

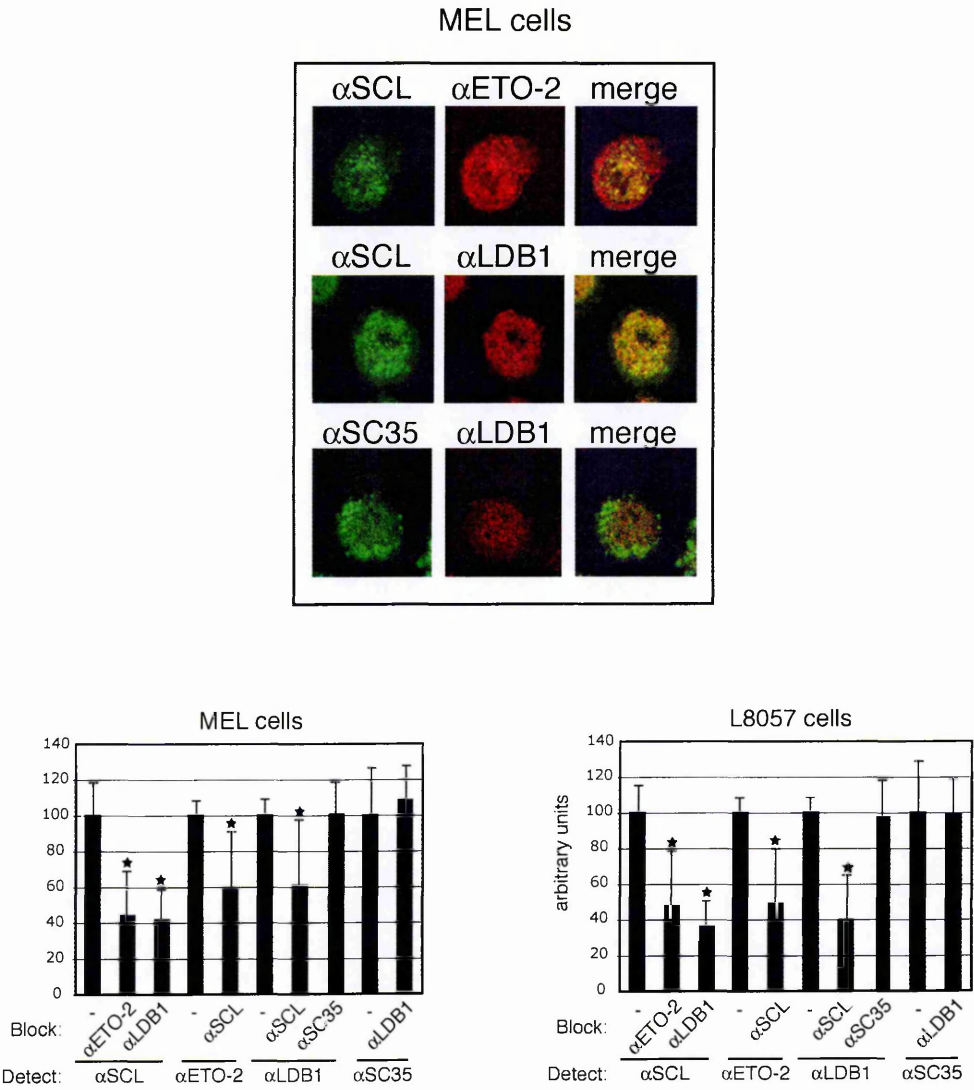


Figure 6.5 Colocalisation
Nuclear co-localisation of SCL with ETO-2 (upper panel, first row) was detected by immunofluorescence using α ETO-2 and α -SCL antibodies. α LDB1 and α SC35 antibody served as a positive control (second row) or negative control (third row), respectively. In an attempt to further validate these results, data of optical intensities over the nucleoplasm of MEL (lower panel, left) and L8057 cells (lower panel, right) were exported to Excel for background subtraction and analysis.
* = p-value < 0.05

localise in the nuclei of both MEL (figure 6.5, top panel) and L8057 (data not shown) cells. As controls, we also show co-localisation of SCL and known partner Ldb1, but not of an abundant unrelated nuclear protein (splicing factor SC35^{210,312}) with a component of the SCL complex such as Ldb1 (Figure 6.5, top panel). Dr Iborra then analysed the results in a different way and used a high resolution approach that takes advantage of the ability of an antibody to block access of another antibody to its antigen (although blocking is incomplete as we demonstrated co-localisation in the merged images, figure 6.5, top panel). In contrast to the conventional analysis indicating that two targets lie within 200nm, this approach reveals targets that lie within a few nanometers^{266,313}. In the absence of blocking antibodies, the intensity of the fluorescence detected in MEL and L8057 nuclei upon incubation with anti-SCL, -ETO2, -Ldb1 and -SC35 antibodies was arbitrarily set to 100 (figure 6.5, bottom panels). The intensity of the signal from SCL antibodies was significantly reduced when co-incubated with ETO-2 or Ldb1 Abs. So were those from anti-ETO-2 and -Ldb1 Abs when incubated with anti-SCL Abs, confirming, at a very high resolution, that these proteins do interact with each other. No interaction could be detected between Ldb1 and SC35.

Validation in primary erythroid cells and megakaryocytes

To confirm these results in primary cells, we isolated Ter119+ cells from spleen of phenylhydrazine-treated mice and megakaryocytes from adult mouse bone marrows (figure 6.6), and prepared nuclear extracts. From both cell types, coIP with SCL Abs under stringent conditions confirmed presence of the SCL core complex (including SCL, E2A, LDB-1 and LMO2), but failed to detect interaction with GATA-1. Interaction with ETO-2 was confirmed. Reverse co-

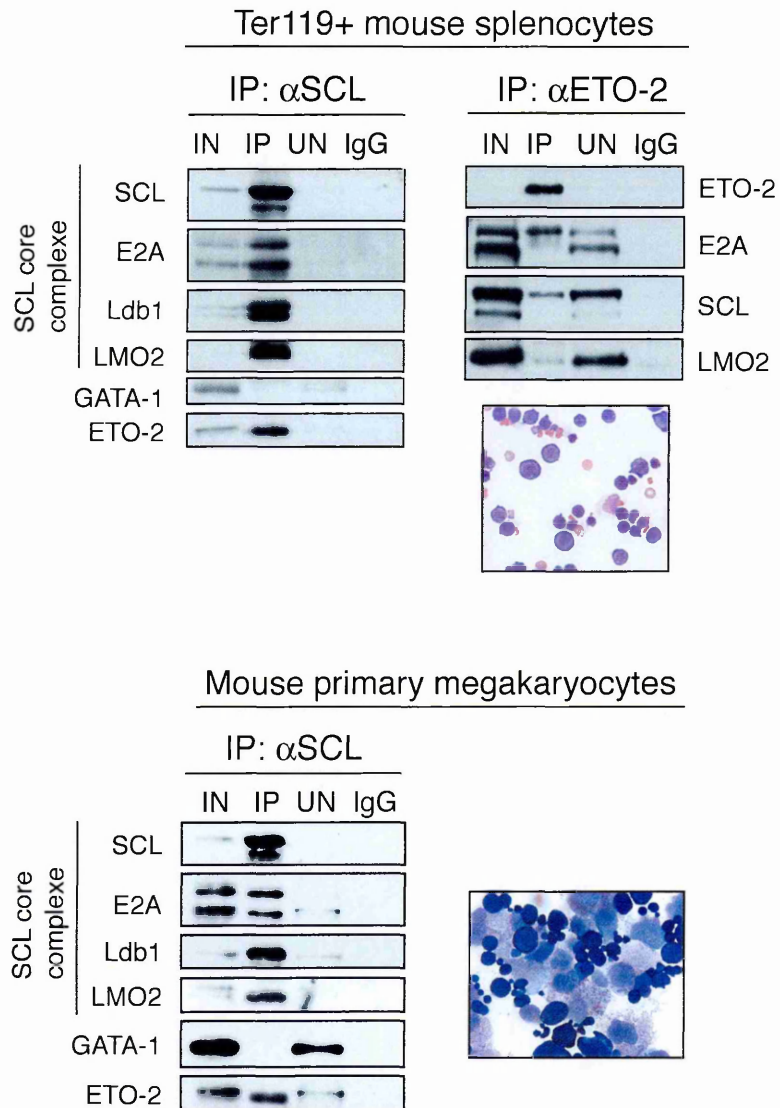


Figure 6.6 ETO-2 interacts with the SCL core complex in primary erythroid cells and megakaryocytes

Nuclear extracts prepared from Ter 119+ mouse splenocytes (upper panel) and primary megakaryocytes (lower panel) were subjected to co-immunoprecipitation with anti-SCL or anti-ETO-2 antibodies (splenocytes only).

The morphology of cells used for nuclear extract preparation was assessed by May Grunwald Giemsa Staining (shown next to each panel).

Ter119+ cells represent proerythroblasts to mature erythrocyte and enucleated stages.

Primary megakaryocytes=immature megakaryocyte precursors (dark cytoplasm) to mature megakaryocytes (large cells with granular cytoplasm and polyploid nuclei).

immunoprecipitation with ETO-2 antibodies from Ter119⁺ extracts confirmed interaction with the SCL core complex (E2A, SCL and LMO2, Figure 6.6).

Taken together, these results validate the interaction between SCL and ETO-2 in wt non-transfected MEL and L8057 cells and reveal the presence of an *in vivo* multiprotein complex comprising the SCL core complex and ETO-2 in primary erythrocytes and megakaryocytes.

3.2 Composition of SCL- and ETO-2-containing complexes differs in erythroid cells and megakaryocytes

In an attempt to finely characterise the nature of SCL-and ETO-2-containing protein complexes, we performed immunodepletion experiments (see scheme figure 6.7A). To do this, nuclear extracts from MEL and L8057 cells were first subjected to immunoprecipitation with antibodies against ETO-2 and GATA-1. After characterisation of the nature of the complexes pulled-down by Western Blot, the depleted supernatants were then subjected to another IP with antibodies against SCL to identify the remaining complexes (figure 6.7B and C).

Immunoprecipitation with ETO-2 antibodies from L8057 and MEL nuclear extracts confirms the interaction of ETO-2 with the SCL core complex observed in primary erythroid cells and megakaryocytes (figure 6.7B, top panel, IP lanes). In MEL cells, ETO-2 was also found to interact with GATA-1. Binding of GFI-1B was also confirmed. Importantly, IP with GFI-1b Abs precipitated SCL from MEL nuclear extracts (figure 6.7D, upper panel) indicating that GFI-1b is also likely to be part of the SCL core complex. It was shown previously that the ETO-2 homolog ETO binds to mSin3A and other co-repressors upon co-expression in heterologous cells¹³¹. Besides, mSin3A has also been known to interact with SCL²²⁰. Therefore, we tested whether we could detect mSin3A in co-immunoprecipitations using

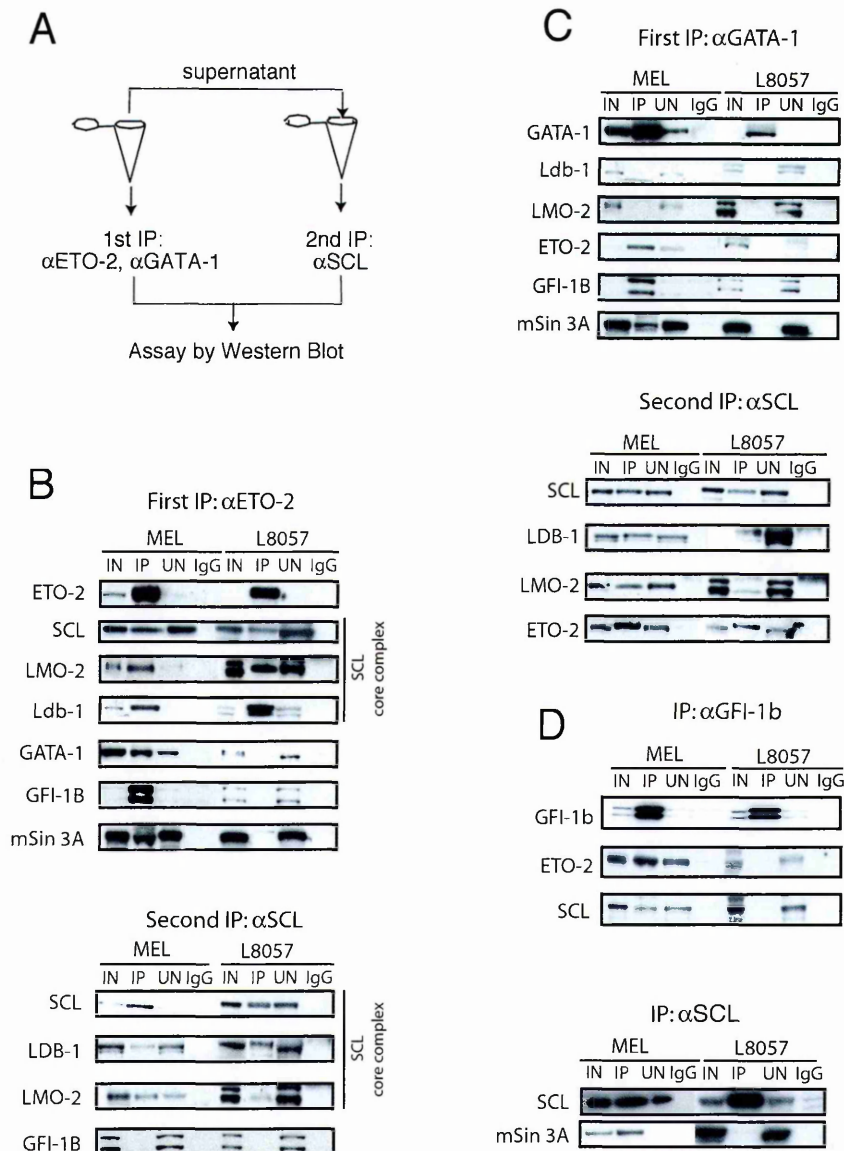


Figure 6.7 Depletion Experiments and further Co-immunoprecipitations

A Scheme of the immunodepletion experiments

B,C Immunodepletion experiments

Wild-type MEL and L8057 cell nuclear extracts were immunodepleted of ETO-2 (B) or GATA-1 (C). Immunoprecipitated complexes were analysed by Western Blot (B and C, upper panels). A second co-IP was performed on the depleted supernatant using α SCL antibodies to analyse the nature of the remaining complexes (B and C, lower panels). The extent of the depletion was assessed by Western Blot with α ETO-2 (B) or GATA-1 (C). Note, that all depletions apart from GATA-1 depletion in MEL cells were complete as no products were detected in the unbound (UN) fractions.

D Co-immunoprecipitation from MEL and L8057 cells with α GFI-1b (top) or α SCL antibodies (bottom).

IN=input of crude nuclear extract, IP=immunoprecipitation, UN=unbound, IgG=negative control.

α ETO-2 (figure 6.7B) and α SCL (figure 6.7D, lower panel). We found that mSin3A interacts with ETO-2 and SCL, but not N-CoR although described previously as a partner of ETO-2¹³⁴(data not shown).

The second IP with SCL antibodies, after depletion of ETO-2, showed that the core SCL complex (SCL, LMO-2, LDB- 1) was present in the absence of ETO-2, and that there was no interaction with Gfi-1B, suggesting that Gfi-1B binds SCL through ETO-2 (figure 6.7B, bottom panel).

Turning to the IP with GATA-1 antibodies under stringent conditions, SCL (data not shown), Ldb1 and LMO-2 were undetectable in the IP fractions (figure 6.7C), confirming the weakness of the interaction between GATA-1 and the SCL core complex. In contrast, there was enrichment of ETO-2, GFI-1B and mSin3A in the precipitated products. The colP on the depleted supernatant with SCL antibodies shows presence of the core complex comprising ETO-2 (figure 6.7C, lower panel). This complements the data obtained upon colP with ETO-2 Abs and shows that the SCL/ETO-2 binding is independent of GATA-1 and that the ETO-2 complex comprising the co-repressor proteins mSin3A and Gfi-1b also involves GATA-1.

In L8057 cells, surprisingly, ETO-2 does not interact with GATA-1 or GFI-1B (figure 6.7B and data confirmed by reverse colP, figure 6.7C (GATA-1)and 6.7D (GFI-1b)) or mSin3A (figure 6.7B), but only with the SCL core complex. The second IP with α SCL shows, as in MEL cells, presence of the SCL core complex in absence of ETO-2. A second IP with SCL Abs from GATA-1 depleted supernatant confirms the presence of the core SCL complex and ETO-2 in the absence of GATA-1 (figure 6.7C, lower panel). In order to exclude that the absence of the SCL/GFI-1b interaction was due to a mechanism specific to the L8057 cell line, we performed

co-immunoprecipitations from nuclear extracts of primary megakaryocytes with α SCL. This confirmed that, in primary megakaryocytes, SCL does not interact with GFI-1b.

The data presented here are summarised in figure 6.8. The model shows a possible representation of the nature of the complexes according to the results obtained from the depletion experiments with the limitations associated to this technique. We cannot exclude the existence of additional complexes and suspect that the proteins represented in a given complex may not always interact altogether.

Taken together, these results led us to the conclusion, that the nature of the SCL and ETO-2 complexes is fundamentally different in erythroid cells versus megakaryocytes.

3.3 Class II bHLH proteins heterodimerise with E2A to bind ETO-2

To map the domain of SCL involved in the interaction with ETO-2, we expressed truncated, biotinylated forms of SCL: (Δ Nt, Δ Ct and Δ Nt- Δ Ct) in MEL cells (figure 5.3). Upon nuclear extract preparation, protein complexes were pulled-down with streptavidin beads and presence of ETO-2 analysed by Western Blot. The bHLH domain of SCL alone was sufficient for interaction with ETO-2 (data not shown). We then analysed binding of ETO-2 to SCL variants that were defective in DNA-binding (SCL-RER)¹⁸⁸ or impaired for interaction with LMO2 (SCL H2(F-G)). SCL-RER was able to bind ETO-2 in both MEL and L8057 cells (figure 6.9 A), suggesting that SCL and ETO-2 may reside in a complex that does not bind DNA, or that binds through the DNA-binding domain of other transcription factors. In MEL cells, interaction between bio-H2(F-G) and ETO-2 are preserved, suggesting that ETO-2 is not recruited to the SCL complex through LMO2/LDB-1. In L8057 cells

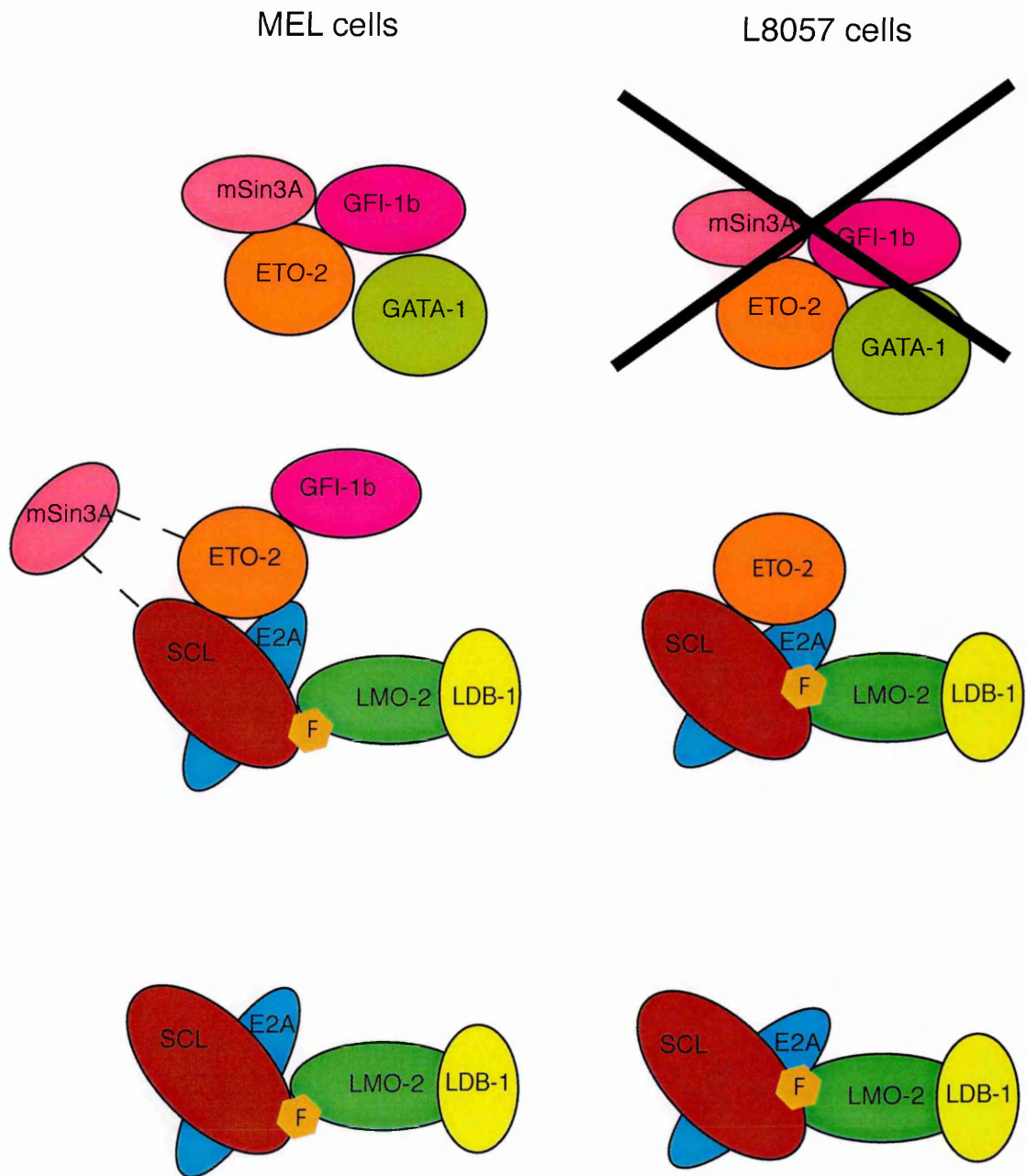


Figure 6.8 Hypothetical model of SCL and ETO-2 containing complexes in erythroid cells and megakaryocytes

This model represents the possible nature of the complexes according to the results obtained from the depletion experiments with the limitations associated to this technique.

We assumed that SCL binds to its heterodimerisation partner E2A.

F=phenylalanine

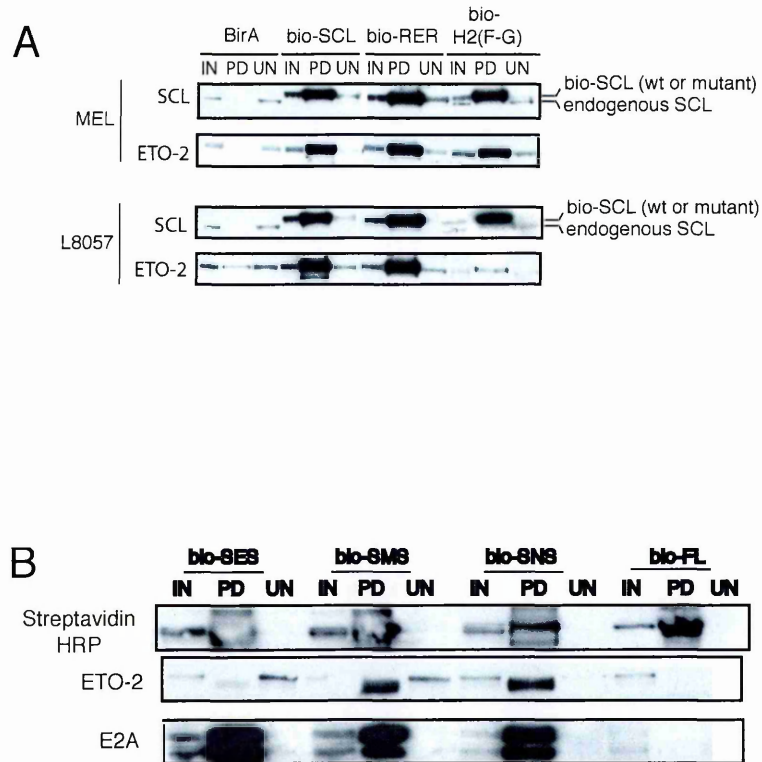


Figure 6.9 SCL mutant analysis

A Nuclear extracts were prepared from MEL (upper panel) and L8057 (lower panel) cells transfected with Bir A only or bio-SCL (wild-type), bio-RER or bio-H2(F-G) and subjected to streptavidin pull-downs. Western Blots were probed with α SCL or α ETO-2 antibodies.

B Nuclear extracts prepared from MEL cell transfected with bio-SES, bio-SMS, bio-SNS and bio-FL were subjected to streptavidin pull-downs. Western Blots were probed with streptavidin-HRP to reveal the mutant SCL or with α ETO-2 and α E2A.

however, H2(F-G) fails to bind to ETO-2 (figure 6.9 A), but also to any of the SCL partners tested, including the heterodimerisation partners E2A and HEB (figure 5.10).

We also generated swapped mutants whereby SCL HLH domain was substituted by the corresponding region of other bHLH proteins (MyoD, E47 and NSCL to produce bio-SMS, bio-SES and bio-SNS, respectively). These variants were able to heterodimerise with E12/E47 as we previously reported ¹⁹³ (figure 6.9 B). Interestingly, whereas substantial amounts of ETO-2 co-purified with the bio-SMS and bio-SNS, interaction with the bio-SES was much weaker (figure 6.9 B). Finally, no interaction between heterodimerisation-defective SCL (SCL-FL) and ETO-2 was detected.

3.4 ETO-2 represses the activator function of the pentameric complex in heterologous cells

To get insight into the possible function of the SCL/ETO-2 complex, we performed transactivation studies of human GATA-1 regulatory sequences in a reporter assay. We recently showed by chromatin immunoprecipitation (ChIP) assay that the DNaseI hypersensitive sites situated 3.5 kb upstream of GATA-1 promoter (hHS-3.5) and 14 kb downstream of GATA-1 promoter (hHS+14) bind the SCL pentameric complex in MEL cells²³¹. These sequences together with human GATA-1 promoter (IE) were used to drive expression of the luciferase gene (construct hHS-3.5-IE-hHS+14) in NIH3T3 cells upon co-transfection with various combinations of vectors expressing members of the pentameric complex (SCL, E47, LMO2, LDB-1 and GATA-1). We show a 7-fold increase in luciferase levels upon co-expression of the five proteins (figure 6.10). In accordance with the ChIP data showing co-occupancy of GATA-1 regulatory regions by these proteins ²³¹,

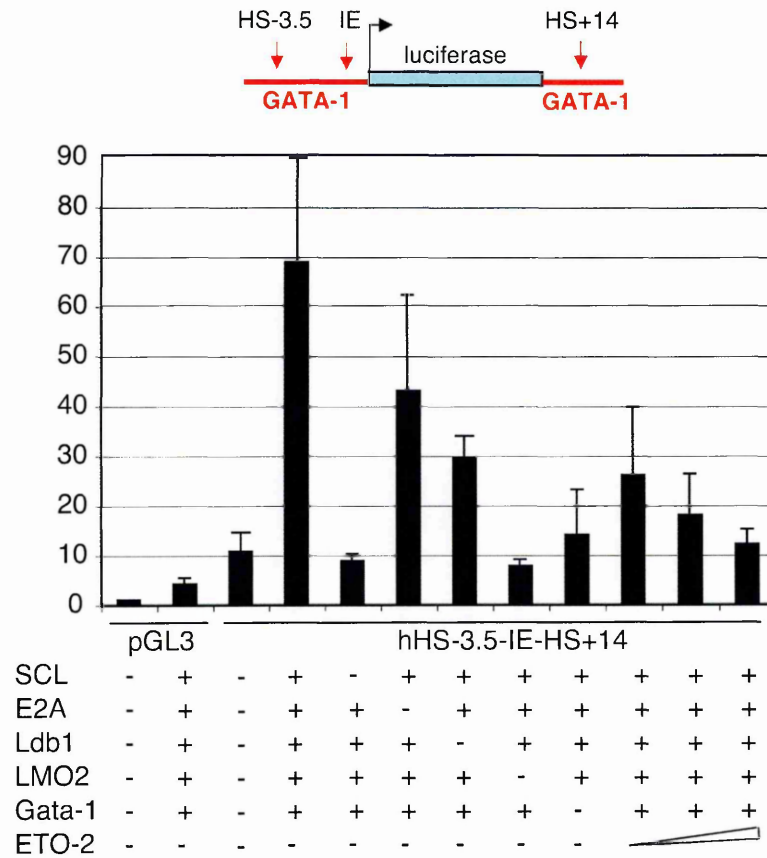


Figure 6.10 ETO-2 represses the activator function of the pentameric complex in transactivation assays

The heterologous cell line NIH3T3 was transiently co-transfected with luciferase reporter genes (see schematic presentation at the top) and vectors expressing the indicated transcription factors: SCL, E2A, Ldb1, LMO2, Gata-1 and ETO-2.

Triangle represents increasing concentrations of ETO-2 expression vector.

Results are shown as the mean \pm standard deviation of three to five experiments performed in triplicate.

pGL3: promoterless; hHS-3.5-IE-HS+14: under control of the promoter/enhancer regions of human GATA-1

each of these factors was found necessary to achieve maximum activation, as expression levels decreased when they were individually omitted. We then co-transfected increasing concentrations of an ETO-2 expression plasmid together with the five components of the pentameric complex. We observed a significant reduction of the levels of activation of the reporter gene and concluded that ETO-2 represses the activator function of the SCL complex in this setting.

3.5 The SCL/ETO-2/GFI-1b interaction is lost during terminal erythroid differentiation

Taken together, these results led us to the hypothesis that ETO-2 might mediate repression of SCL target genes which have to be silenced for differentiation to proceed normally. In a first attempt to test this hypothesis, we established an expression profile of proteins involved in the SCL/ETO-2 interaction in non-induced and induced MEL cells (figure 6.11 A). This revealed that expression of ETO-2 is significantly reduced in induced MEL cells, whereas protein levels of the other proteins remain stable or increase slightly. This suggests that the repressor function of SCL may be alleviated upon silencing of ETO-2 expression during erythroid differentiation. As induction of MEL cells might not precisely mirror erythroid differentiation, we decided to test our hypothesis in a different system and performed *in vitro* differentiation of day 12.5 primary fetal liver cells³¹⁴(figure 6.11B).

To examine how a decrease in ETO-2 protein levels during erythroid differentiation could impact on the SCL-containing complexes, we analysed their composition during *in vitro* erythroid differentiation of fetal liver cells. We isolated a pure population of undifferentiated, benzidine-negative, c-kit⁺, Ter119⁻ erythroid

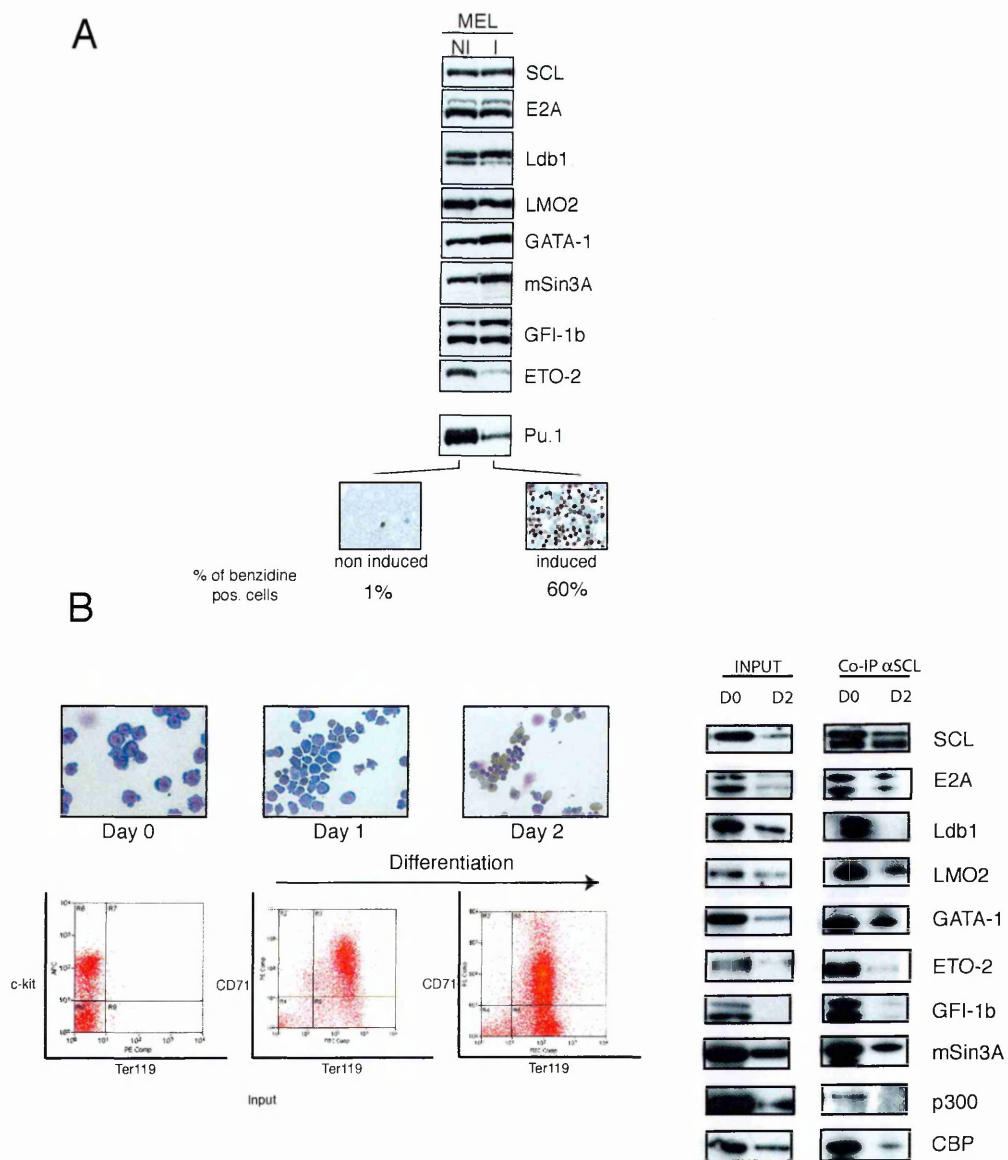


Figure 6.11

A: Expression levels of indicated proteins in non-induced and induced MEL cells. Western Blot analysis of nuclear extracts prepared from non-induced=NI and induced=I MEL cells. (Decrease of levels of expression of PU.1 serves as a control of induction of MEL cell terminal maturation).

B: *In vitro* erythroid differentiation of day 12.5 wild-type primary fetal liver cells.

Benzidine staining (left upper panel) and FACS analysis (left lower panel) of cells after 0, 1 and 2 days of differentiation.

Right panel: Expression profile (left) and co-immunoprecipitation (right) of proteins as indicated using nuclear extracts prepared from fetal liver cells on day 0 and day 2 of differentiation.

precursors (Day 0) that were subjected to differentiation to obtain terminally differentiated, benzidine-positive CD71+, Ter119+ erythrocytes (Day 2). Western blot analysis of these populations showed a decrease in levels of all the proteins we tested upon differentiation (figure 6.11, right panel, INPUT). In erythroid precursors (Day 0), SCL interacted with all of its known partners including GATA-1 and with ETO-2, GFI-1b and mSin3A. However, in terminally differentiated erythrocytes (Day 2) the interaction with ETO-2 and GFI-1b was lost although SCL still bound to the members of the core complex (except for LDB-1). Interestingly, α SCL also co-immunoprecipitated mSin3A, suggesting that mSin3A binds to SCL independently of ETO-2 and GFI-1b.

The co-activator and intrinsic histone-acetylase CBP/p300 was previously shown to interact with SCL¹⁹⁸. Besides, supporting the idea of a co-factor exchange, recent work by Zhang et al¹³⁹ suggests that in HeLa cells, E2A can act both as a repressor or activator by exchanging members of the ETO family for the co-activator CBP/p300. We therefore tested the hypothesis that CBP/p300 binding to SCL might increase in terminal differentiation. However, in our system in primary erythroid cells, SCL/CBP/p300 containing complexes decreased with differentiation.

In summary, we therefore conclude that SCL binds to ETO-2 and GFI-1b primarily in erythroid precursors and that this interaction is lost in terminal differentiation whereas mSin3A continues to bind to SCL. Furthermore, we show that in terminal erythroid differentiation, the mechanism underlying ETO-2 directed repression is not the inhibition of co-factor exchange with CBP/p300.

4 DISCUSSION

Using a novel protein purification approach, we have identified previously unknown partners of SCL and show that SCL interacts with SSDP2 and the co-repressor ETO-2 in erythroid cells and megakaryocytes.

SSDP2 has been described as a co-factor of LDB-1 and LMO-2 orthologs in the early development of drosophila and xenopus (see above). Here, we show for the first time that SSDP proteins are also involved in the formation of haematopoietic multiprotein complexes in vertebrates. This is of particular interest as genes of the *ssdp* family have been localised to translocation breakpoints and deletions in myeloid malignancies³¹⁵.

ETO-2 came up consistently in our mass spectrometry analysis as a partner of SCL and the interaction between the two endogenous proteins was characterised using a variety of different approaches.

Co-localisation

Due to its low resolution of several hundred nanometres, the use of conventional co-localisation for transcription factors and other small molecules has been questioned. Therefore, we opted for an additional and previously validated technique to analyse optical intensities from co-localisation experiments²⁶⁶, and show that SCL and ETO-2 co-localise within only a few nanometers from each other.

Immunodepletions and co-immunoprecipitations

In order to further characterise the nature of the SCL/ETO-2 containing complexes, we performed depletion experiments and further co-immunoprecipitations. These

experiments allowed us to start to define the composition of the complexes and to identify other previously unknown partners.

mSin3A

ETO-2 interacts with mSin3A in MEL cells. This is in contrast to recently published data, showing that ETO, but not ETO-2 could interact with mSin3A upon co-expression in heterologous cells¹³⁴. This difference may reflect the difference in cell types used in this study and ours (erythroid cell line versus Cos 7 cells) and/or in the levels of expression between endogenous and overexpressed proteins. Alternatively, ETO-2 may be in the same complex with mSin3A, but not physically binding to it.

It is of note that, in accordance to previously published data²²⁰, we, too, show that mSin3A binds to SCL. Thus, mSin3A might also interact with SCL independently of ETO-2. This idea is consistent with our preliminary data from immunodepletion experiments using nuclear extracts prepared from the T-cell leukaemia cell line Jurkat. These experiments reveal the presence of a complex containing SCL and mSin3A after immunodepleting with α ETO-2.

GFI-1b

Our data indicate that SCL and ETO-2 co-migrate with GFI-1b and that the three proteins co-immunoprecipitate in MEL cells and primary erythroid precursors, but not in megakaryocytes. Although it was recently shown that GFI, a GFI-1b homolog, interacts with ETO³¹⁰, we show here for the first time that GFI-1b and ETO-2 bind to an essential sequence specific transcription factor (SCL) in erythropoiesis.

The gene locus encoding for the growth independence 1 (Gfi) protein was first discovered in a screen for Moloney murine leukaemia virus proviral integration

sites in T cell lymphoma³¹⁶. Gfi-1 zinc finger protein and its homologs have been implicated in processes as diverse as oncogenesis³¹⁷, apoptosis^{316,318,319}, proliferation³²⁰, cell fate specification and differentiation^{102,311}. Studies in vertebrates have focused mostly on haematopoietic lineages whereas research with *C elegans* and *Drosophila* has centred on the nervous system. Given their structural similarities, it is reasonable to think that the two proteins might perform redundant functions, although growing evidence suggests that they are not always interchangeable^{321,322}. Gain-of-function studies have indicated a role for GFI-1b in terminal erythropoiesis³²³. Besides, loss-of-function studies have established that Gfi-1B plays an essential role in the generation of red blood cells and platelets³¹¹. Gfi-1B mutant embryos form immature primitive erythrocytes, but definitive erythropoiesis is disrupted, leading to death at dpc 15. Hence, in the absence of Gfi-1B, haematopoietic progenitors commit to the erythroid lineage but fail to mature. Similarly, there is a block in megakaryocytopoiesis. Lack of Gfi-1 on the other hand severely affects lymphoid and granulocyte development^{102,318}.

Gfi-1 and its vertebrate homolog Gfi-1B act as transcriptional repressors via their Snail/Gfi-1 (SNAG) domain³²⁰ and can bind directly to DNA. Besides, Gfi-1 can function as a transcriptional repressor even in the absence of the SNAG domain³¹⁰. In this case, Gfi-1 requires protein-protein interaction with HDACs and the co-repressor ETO. Interestingly, recent data suggests that GFI-1b also acts as a repressor during human erythroid differentiation³²³. Moreover, Gfi-1 and Gfi-1B can activate transcription in an erythroid cell line³²⁴, hence implying that they affect target promoters differentially depending on the cellular context.

Importantly, Gfi-1 and its homologs seem to cooperate with bHLH transcription factors in different tissues, including T cells³²⁵ and T cell lymphoma³¹⁹, although a

direct physical interaction has not been described so far. In view of their similar expression profiles and knock-out phenotypes a possible interaction between SCL and GFI-1b seemed plausible, but had not been formally demonstrated so far.

GATA-1

Interestingly, in erythroid cells, we found a complex consisting of ETO-2, Gfi-1b, mSin3A and GATA-1, but without the SCL core complex. This is consistent with results obtained in primary splenocytes and megakaryocytes, where we were unable to show SCL/GATA-1 binding. The reasons for this may be threefold

1. The importance of stringency conditions when performing affinity purifications and co-immunoprecipitations has been stressed before. A fine balance between preserving protein-protein interactions, but eliminating non-specific background binding has to be kept. Immunodepletions and co-IPs on primary cells were performed under stringent conditions. As mentioned previously, this may explain why the GATA-1/SCL interaction could not be revealed in these experiments.
2. Besides, the SCL/GATA-1 complex may be less abundant than the SCL core and the GATA-1/Gfi-1b/mSin3A/ETO-2 complex.
3. Alternatively, if there is indeed a bias towards preferentially purifying repressor complexes with this technique, this may also indicate that the SCL/GATA-1 interaction is part of an activating complex.

Taken together, although incomplete, the immunodepletion experiments allowed us to conceive a hypothetical model of the nature of the complexes within the limitations associated to this technique. The principle problem we encountered in interpreting the results was directly related to the complexity of the cellular protein

network. When performing co-immunoprecipitations of transcription factors, results obtained always derive from a pool of different cells at different stages of their cell cycle and different genes in repressed or active states. Hence, at any one time, a whole spectrum of protein complexes may be present in the nuclear extracts used. Besides, we cannot exclude the existence of additional complexes and suspect that the proteins represented in a given complex may not always interact altogether. However, from these experiments and the subsequent validations in primary erythroid cells and megakaryocytes, it has become apparent, that the SCL/ETO-2 complexes in these two cell types are fundamentally different. In red cells, the SCL core complex interacts with ETO-2, GFI-1b and mSin3A, whereas in megakaryocytes, the SCL core only binds to ETO-2.

SCL mutants

Analysis of the SCL mutants revealed that the DNA binding activity of SCL is dispensable for the interaction with ETO-2, indicating that the complex could either form off DNA or via DNA binding of other members of the complex. Mapping of the interaction domain confirmed that the bHLH domain of SCL is sufficient for ETO-2 binding. This is not unexpected, as it was shown previously that the bHLH domain is sufficient for all of SCL's functions¹⁸⁸ including heterodimerisation with its partner E2A, and that ETO-2 binds to E2A via a short aminoacid sequence at the N terminus of E2A¹³⁹. In the same study, E2A homo- and heterodimers transfected into HeLa cells bound ETO-2 equally well. Our data from MEL cells reveals that, although all SCL swapped mutants heterodimerise with E2A, only SMS, containing the MyoD HLH domain and SNS (for NeuroSCL HLH domain) but not SES (for E2A HLH domain) bind to ETO-2. This may indicate that bHLH heterodimers have a stronger affinity to ETO-2 than E2A homodimers and that the tissue specific class

II bHLH protein is necessary to recruit the repressor complex to specific target genes, whereas E2A serves as the ubiquitous anchor protein.

ETO-2/SCL may act as a repressors of gene expression in early erythroid differentiation

Previous data have shown that ETO proteins can act as co-repressors of gene expression. We performed transactivation studies, which confirmed the repressive effect of ETO-2 on the activator function of the pentameric complex. Moreover, using *in vitro* differentiation of fetal liver cells, we could show, that the SCL/ETO-2/GFI-1b interaction is lost with erythroid differentiation, whereas the SCL core complex also comprising GATA-1 remains intact. Interestingly, we did not see a decrease in SCL/mSin3A containing complexes, further indicating, that mSin3A may bind to SCL independently of ETO-2 and Gfi-1b.

Taken together, it is possible that ETO-2 is required in the early stages of erythroid differentiation to repress expression of target genes of SCL. Down-regulation of its expression then leads to the release of the repressive status and to gene activation, therefore allowing terminal erythroid maturation.

This repression may be mediated by recruitment of HDACs. Our preliminary data suggests, that ETO-2 and SCL indeed interact with HDAC-3 in MEL cells (data not shown). On the other hand, ETO-2 could prevent interaction of SCL with a co-activator. Co-factors exchange is a commonly used mechanism to regulate levels of transcriptional activation. Different classes of transcription factors can recruit both co-repressors and co-activators ^{115 116} as recently exemplified by NF-E2p18/MafK whose dimerisation partner switches from co-repressor to co-activator during MEL cell differentiation ¹¹⁷.

Moreover, recent data also suggests that binding of ETO to E2A might lead to dissociation of the E2A/p300 interaction¹³⁹. However, we could not confirm that SCL/CBP/p300 containing complexes increased with terminal erythroid differentiation and concluded that co-factor exchange involving CBP/p300 was not the mechanism underlying ETO-2 mediated repression of gene activation in this setting. Finally, ETO-2 could sequester the SCL core complex and prevent its binding to DNA.

In order to pursue the functional characterisation of the SCL/ETO-2 interaction in haematopoiesis, we have taken both gain-of-function and loss-of-function approaches. Thus, we are currently investigating the effects of over-expression of ETO-2 or E2A mutants that are unable to bind ETO-2¹³⁹ on the *in vitro* haematopoietic differentiation of wild-type ES cells.

FINAL DISCUSSION AND FUTURE DIRECTIONS

The aim of this work was to further define the role of the bHLH transcription factor SCL in definitive haematopoiesis and leukaemogenesis. We began with the phenotypic characterisation of a transgenic mouse model over-expressing SCL under the control of the *sca-1* promoter sequences. Ectopic expression of SCL in thymocytes and B-lymphocytes led to a profound disturbance of the normal differentiation program in these cells although no leukaemia was observed. These findings led us to the conclusion that SCL sequestered E2A proteins essential for lymphocyte development and that SCL on its own was only weakly oncogenic. This, together with other lines of evidence already mentioned above, led us to investigate the importance of protein-protein interactions for SCL's functions, and we were able to identify novel SCL partners in erythropoiesis and megakaryocytopoiesis. It now seems likely, that SCL can exert both activating and repressive function on its target genes depending on the cell type and the stage of differentiation examined. We established that the repressor effect in early erythroid differentiation is mediated by a co-repressor complex containing ETO-2 and GFI-1b and that the underlying mechanism is probably not co-factor exchange involving CBP/p300.

We now plan to further characterise the role and mechanism of this interaction in erythroid differentiation and leukaemogenesis. We have generated MEL cell clones containing biotinylated SSDP2, E2A and ETO-2. These will allow us to further elucidate the multi-protein network surrounding SCL. Besides, we will attempt to generate transgenic mice expressing a biotinylated version of SCL. This will enable

us to perform pull-downs on specific cell populations after cell sorting. Moreover, it will be critical to find new SCL target genes that might also recruit ETO-2. However, one of the principle questions to be addressed remains the exact role and mechanism of the ETO-2/SCL interaction in haematopoiesis. To this aim, loss-of-function studies of ETO-2 such as siRNA or morpholino will address the role of this widely expressed protein in general. In addition, in order to specifically target the SCL/ETO-2 complex, it will therefore be essential to identify small molecules or peptides inhibiting this interaction. Several publications have recently successfully attempted this approach to characterise protein-protein interactions. Using the information obtained from the crystal structure of the respective proteins, it has been possible to engineer stable peptides that mimic BAD³²⁶ or interfere with the BCL 6 interaction³²⁷. The design of such molecules will not only allow us to answer the fundamental questions surrounding protein-protein interactions, but may also represent a new approach for future therapeutic drug targeting.

As far as leukaemogenesis is concerned, our preliminary data suggests that ETO-2 is expressed in the SCL expressing acute T-cell lymphoblastic leukaemia cell line Jurkat and that it binds to SCL in this cell type.

Hence, in addition to sequestering E proteins, SCL could recruit repressor complexes to genes normally switched on in differentiating T cells. Two papers now suggest that both these mechanisms may be operative. Herblot et al demonstrated that SCL and LMO-2 overexpression in mice leads to inhibition of E2A/HEB function and repression of the pre-T α gene expression, essential for normal T cell development¹⁴⁹. Consistent with this, O'Neil et al showed that expression of an SCL transgene in an E2A or HEB heterozygous background promotes development of leukaemia. Using ChIP assays, they demonstrated that

the SCL/E2A heterodimer recruits the co-repressor complex mSin3A/HDAC1 to enhancer elements of genes important for thymocyte differentiation, such as CD4 and pre-T α ²⁵⁶.

Taken together, these data indicate that, in T-ALL, SCL may form aberrant protein complexes, therefore perturbing expression of genes essential for normal T-cell differentiation.

However, these aberrant SCL-containing complexes remain to be fully characterised and their exact molecular mechanism of action elucidated. Moreover, their relevance in human leukaemogenesis needs to be investigated.

In conclusion, we are only beginning to understand how the cross-talk between sequence-specific transcription factors and their co-factors regulates gene transcription. After the discoveries of genetic code and histone code, study of the composition and dynamics of multimeric transcription factor complexes and their effects on gene regulation may lead us to the identification of a « transcription factor complex code ». Decrypting this code will shed further light on the fundamental questions of how gene transcription is controlled and will enable us to envisage novel therapeutics for our patients.

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